UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE AGRONOMIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA DO SOLO

MECANISMOS DE ESTABILIZAÇÃO DE CARBONO EM ARGISSOLO SUBTROPICAL SOB SISTEMAS DE MANEJO DE LONGA DURAÇÃO

Murilo Veloso Gomes

(Tese)

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MECANISMOS DE ESTABILIZAÇÃO DE CARBONO EM ARGISSOLO SUBTROPICAL SOB SISTEMAS DE MANEJO DE LONGA DURAÇÃO

Autor: Murilo Veloso Gomes

Orientador: Cimélio Bayer

Resumo

No Brasil, o acúmulo de carbono orgânico no solo (COS) tem sido obtido em plantio direto (NT), que faz parte do plano de agricultura de baixo carbono para a mitigação da emissão de gases do efeito estufa. Quando combinado com leguminosas de cobertura, o NT pode resultar ainda em maiores taxas de acúmulo de COS. Baseado num experimento de 30 anos, seis estudos foram realizados com o objetivo geral de avaliar o efeito de dois métodos de preparo (preparo convencional –CT e NT) com três sistemas de culturas [aveia/milho (O/M), ervilhaca/milho (V/M) e o consórcio aveia+ervilhaca/milho+caupi (OV/MC)] sobre os mecanismos de estabilização do C e consequente potencial de sequestro de COS em camadas superficias e subsuperficias de um Argissolo subtropical do Sul do Brasil. O primeiro estudo engloba uma meta-análise global avaliando o potencial do NT em acumular COS. Os resultados deste estudo mostraram um grande potencial de acumular COS em regiões tropicais e subtropicais, o que foi explicado pela alta precipitação média anual nessas regiões. O segundo e terceiro estudos mostraram um importante mas diferenciado papel do plantio direto e das leguminosas de coberturas sobre a estabilização do C no solo, sendo que o não revolvimento favoreceu a oclusão da matéria orgânica em macroagregados enquanto que as leguminosas favoreceram a associação organomineral em microagregados. Neste estudo, a importância dos macroagregados sobre a associação organomineral foi enaltecida. Além disso, o quarto estudo revelou forte contribuição das leguminosas de cobertura sobre o potencial de acumular COS em NT, sendo que altas taxas de acúmulo de COS ocorreram durante um período mais longo em camadas subsuperficias do solo. No quinto estudo, uma combinação de fracionamento densimétrico e granulométrico com análises de carboidratos e *n*-alcanos foi realizada objetivando maior entendimento do papel das leguminosas de cobertura sobre o acúmulo de C em associações organomineral. Este estudo deu suporte à hipótese que o acúmulo de C adicional na fração argila em camadas superficiais e subsuperficias do NT com leguminosas de cobertura é microbiologicamente processado. No sexto estudo, encontramos que o maior teor de C na fração leve do solo sob plantio direto e leguminosas de cobertura favoreceu a comunidade de fungos que, por sua vez, mediou a melhora na agregação de solo. O acúmulo de constituintes da parede celular de fungos contribuiu, portanto, ao acúmulo de COS e agregação de solo sob plantio direto e leguminosas de cobertura.

[1] Tese de Doutorado em Ciência do Solo. Programa de Pós-Graduação em Ciência do Solo, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul. Porto Alegre (135 p.). Março, 2018. Trabalho realizado com apoio financeiro do CNPq e da CAPES.

MECHANISMS OF CARBON STABILIZATION IN A SUBTROPICAL ACRISOL UNDER LONG-TERM MANAGEMENT SYSTEMS[1]

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Abstract

In Brazil, soil organic carbon (SOC) accumulation have been observed under no-tillage (NT) which is part of a low carbon agriculture plan for mitigation of greenhouse gases emissions. When combined with legume cover crops, NT could result in even greater soil organic carbon (SOC) sequestration rate than NT alone. Using a 30-year experiment, we performed six studies for which the general objective was to evaluate the effect of two tillage (conventional system – CT and NT) with three cropping systems [oat/maize (O/M), vetch/maize (V/M) and the consortium oat+vetch/maize+cowpea (OV/MC)] on stabilization mechanisms of C and consequent SOC sequestration potential in both the superficial and sub-superficial soil layers of a subtropical Acrisol of Southern Brazil. Encompassing a meta-analysis evaluating the potential of NT in accumulate SOC around the world, the first study showed a great potential in tropical and subtropical regions to accumulate SOC that was explained by the high mean annual precipitation. In addition, the second and third studies showed the meaningful but differential roles of no-tillage and legume cover crops on C stabilization with the former favoring occlusion in soil macroaggregates and the latter, mineral-organic association in soil microaggregates. In these studies, we supported the importance of macroaggregates to the mineral-organic association. The fourth study revealed the strong contribution of legume on the potential of SOC sequestration in NT, and high rates of C accumulation occurred over a longer period in sub-superficial soil layers. In the fifth study, we used density and particle size fractionation in combination with carbohydrate and *n*-alkane analyses to provide better understanding of the effect of legume cover crop on C accumulation in mineral-organic association. This study gives support to the hypothesis that the additional clay-bound SOC accumulation at depth under NT with legume cover crops is microbially processed. In the sixth study, we found that the greater C content in light fraction under NT and legume cover crops favoured the fungal community which, in turn, mediated the improvement in soil aggregation. The accrual of fungal cell-wall constituents contributed therefore to SOC accumulation and soil aggregation under NT and legume cover crops.

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1. GENERAL INTRODUCTION

Soil organic carbon (SOC) accumulation is essential for more sustainable and resilient agricultural systems, especially in tropical and subtropical soils, due to its central role in determining soil properties that strongly affect crop production and the quality of the wider environment (Lal, 2009; Powlson et al., 2016). The intense solar radiation and high precipitation in tropics and subtropics enable the cultivation of wide diversity of plants over the year, neverthless they result in high decomposition rates of the SOC (Mielniczuk et al., 2008). Although the favorable environment to microbial decomposition of SOC, management systems can make agricultural soils potential sinks or sources of atmospheric CO_2. In addition, Brazil made a voluntary commitment to reduce greenhouse gas (GHG) emissions by 37% by 2025 and established a climate plan, which encompasses a low carbon agriculture plan (Brazil Ministry of the Environment, 2015).

No-tillage (NT) is the basis of conservation agriculture and is essential to the sustainability of agriculture activities in tropical and subtropical regions (Paustian et al., 1997; Bayer et al., 2000; Lal et al., 2007). In Brazil, NT covers an area of 32 millions of hectares (FEBRAPDP, 2012), and there is a proposed conversion of another 8 million hectares for the coming years (MAPA, 2012). The NT is included as low carbon agriculture plan as one of five thrusts for mitigation of GHG emissions por ser uma das principais estratégias para o sequestro de carbono no solo.

However, the use of cover crops is restricted, and the challenge to improve the cropping systems with cover crops in the winter remain. In Rio Grande do Sul State, more than 50% of area cultivated with summer cops (8.5 millions of

hectares) remain under fallow (CONAB, 2016; IBGE, 2016). Although the benefits of high residue input to SOC accumulation being well known (Zanatta et al., 2007; Vieira et al., 2009), the influence of residue quality on SOC accumulation is still inconsistent. Recent studies have suggested the positive influence of labile residues (labile compound and N) on the amount of microbial product and consequently were stabilized by mineral-organic association (Cotrufo et al., 2015). Thus, the characterization of the stable C source is necessary to predict the effects of amount and quality of the residue on SOC stabilization.

Highly weathered soils composed mainly of kaolinite and oxides of iron and aluminum predominate in tropical and subtropical regions of Brazil. As the soils are predominantly deep soils and under region with high precipitation, management systems can alter soil properties in the deeper layers in the long term. Unlike studies in temperate climate, SOC accumulation below the 20 or 30 cm soil layer have been observed in many studies in the Brazilian Subtropical zone (Figure 1). Thus, further studies are needed to elucidate how these management systems in subtropical soil contribute to SOC accumulation in subsurface.

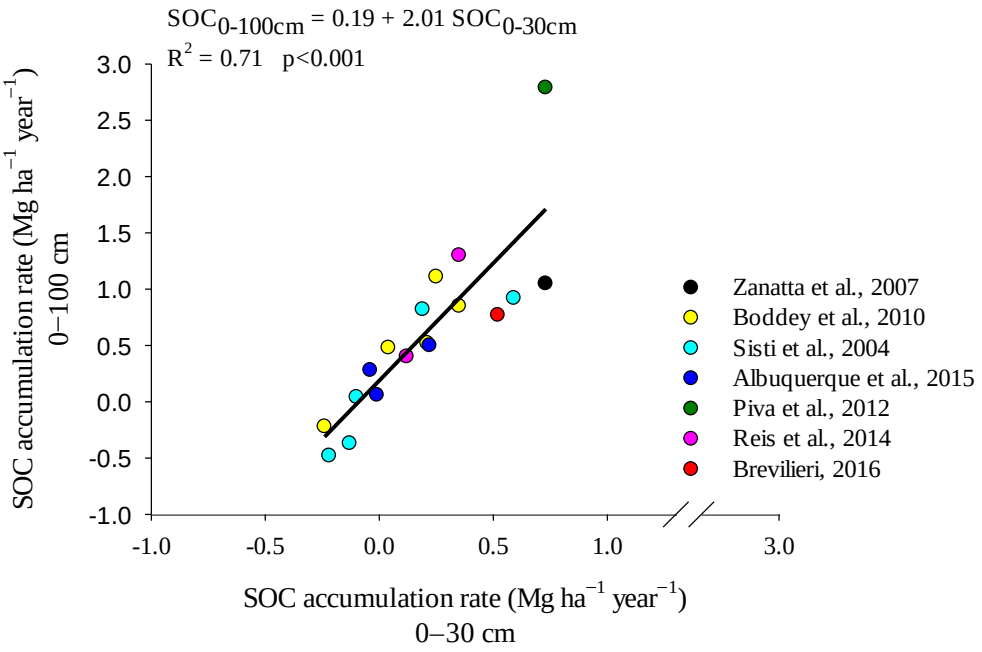

Figure 1. Regression of soil C accumulation rate between 0–30 and 0–100 cm soil layer under different soil and management systems in Southern Brazil.

The physical densimetric fractionation enable to study the relationship between the location and amount of C in the soil structure and might contribute to a better understanding of stabilization mechanisms of SOC (Conceição et al., 2013). Three fractions are obtained: free light fraction (FLL-C), light occluded fraction (Occluded-POM-C) and heavy fraction (min-OM-C). The FLL-C fraction is derived from plants and hyphae residues that still have recognizable cellular structures and the permanence in soil is conditioned to the recalcitrance of its constituent compounds (von Lutzow et al., 2006; Conceição et al., 2013; Plaza et al., 2013). The Occluded-POM-C is stabilized by molecular recalcitrance, but mainly by physical occlusion or protection in aggregates (Conceição et al., 2013) (Conceição et al., 2013). In Min-OM-C, the mineral-organic association, via adsorption, act as a stabilizing mechanism of C (Kogel-Knabner et al., 2008). The LFF-C, Occluded-POM-C and Min-OM-C might have different contributions to SOC according to soil use and management and soil texture and mineralogy (von Lutzow et al., 2006; Yamashita et al., 2006; Zotarelli et al., 2007; Garcia-Franco et al., 2015) and the C might be transferred between fractions (Golchin et al., 1994; Du et al., 2015). The Occluded-POM-C (representing physical protection) and Min-OM-C (representing mineral-organic association) are considered the main mechanisms of C stabilization in soils with predominance of 1: 1 clay and oxides (Denef et al., 2007; Zotarelli et al., 2007; Conceição et al., 2013).

Therefore, the investigation of management systems influence on the stabilization mechanisms of C is relevant for the better understanding of processes and the selection of management practices that favour the SOC accumulation in tropical and subtropical soils. Based on a long-term experiment (30-year) in Southern Brazil on an subtropical Acrisol, we developed six studies with the following objectives:

i) Using meta-analysis based on global data, we related MAP with the potential of NT compared to conventional tillage (CT) to accumulate SOC.

ii) C dynamics in these classes to better understanding the contribution of physical protection and mineral-organic association affected by management systems on mean residence time of C

iii) Investigate the influence of NT and legume cover crops on the fungal or bacterial cell-wall constituents and their potential influence on soil aggregation and organic C forms

iv) Evaluate the SOC sequestration potential of NT and the contribution of legume cover crops and nitrogen (N) fertilization to this potential in both the superficial and sub-superficial soil layers

v) Evaluate the effect of NT and the contribution of legume cover crops and N fertilization on the neutral total sugar and *n*-alkane and organic C (total and in mineral-organic associations) from surface and subsurface soil layers to ascertain the theory that crops with high residue lability associated with NT improve the microbial residues in mineral-organic association, favouring SOC accumulation.

vi) Evaluate the influence of management systems on SOC accumulation, seeking to understand the mechanisms of C stabilization in aggregate classes

2. SOIL CARBON ACCUMULATION UNDER NO-TILLAGE IS DEPENDENT OF SITE-SPECIFIC CLIMATE CONDITIONS

2.1 Abstract

Adopting no-tillage in agroecosystems has been widely recommended as a measure to enhance C accumulation in soils. However, ranges of positive and negative results have been obtained, and the reason for this variability are still unclear. Our hypothesis is that NT system is dependent of site-specific climate conditions to ensure C accumulation. Using meta-analysis based on global data, we related mean annual precipitation (MAP) and mean annual temperature (MAT) with the potential of no-tillage (NT) and conventional tillage (CT) to accumulate soil organic carbon (SOC). In all cases there was a positive effect of NT on SOC in 0–5 cm soil layer. In this soil layer, the difference in SOC accumulation between NT and CT was best fitted in a hyperbolic curve (R^2 = 0.59; $p<0.002$) as a function of MAP. In 0–30 cm soil layer, the SOC accumulation under NT followed the tendency as the 0–5 cm soil layer and was still dependent of the MAP. High accumulation at dry sites (< 500 mm $year^{-1}$) was possibly due to the mulch effect, that leaving the residues at surface slow down their decomposition. Around 700–800 mm $year^{-1}$, the SOC accumulation under NT was around zero or negative, due possibly to high SOC in deeper soil layers under CT, which have compensation effect with the gains that occurred in surface layer. Allied with greater capacity of residue input, the high MAP invoked positive effect of NT in accumulate SOC. We conclude that at the global scale the response of SOC to NT is highly dependent on climate, especially precipitation, which is mandatory on the positive balance between input and decomposition of C.

2.2 Introduction

Tillage systems have been used as strategy to promote SOC accumulation. A large number of studies have shown that the adoption of NT leads to an accumulation of SOC at soil surface (West & Post, 2002). However, others studies have shown no difference or even higher values under FIT, especially when the whole soil profile is considered (Angers & Eriksen-Hamel, 2008). Under FIT, crop residues are distributed uniformly throughout the plow layer, as opposed to NT where they accumulate at soil surface (Balesdent et al., 1990; Angers et al., 1995; Clapp et al., 2000). Consequently, the net effect of FIT vs. NT on total C stocks is sometimes difficult to predict (Angers & Eriksen-Hamel, 2008).

In an investigation of contrasting experimental sites, Helgason et al. (2010) observed that, NT systems in particular require different management strategies as a function of climate in order to manipulate the microbial community for optimal C sequestration. Climatic factors such as precipitation have been shown to exert a control on the potential of NT to store SOC. Greater C sequestration potential in drier than in humid sites have been observed (Blanco-Moure et al., 2013; Dimassi et al., 2014). The temperature has also been shown an important factor for SOC accumulation under NT. The rate of carbon accumulation decreased under high mean annual temperatures, implying that more crop residues should be left on the soil surface to favour the SOC accumulation at high temperatures (Potter et al., 2007).

The specific purpose of this study was to review published data on the influence of NT on SOC content in the soil profile and, determining the factors controlling the occurrence of sequestration below the soil surface under NT.

2.3 Materials e Methods

A data set of long-term tillage studies was compiled from a survey of the scientific literature and included studies from previous tillage literature reviews (Angers & Eriksen-Hamel, 2008). Studies were included in the data set if the

following criteria were met: (i) studies compared NT and CT systems; (ii) studies were replicated experimental designs with the same soil type for both tillage treatments; (ii) the average was calculated for studies with different cropping systems or residue management (iii) SOC was sampled to 0–5±2.5 cm soil layer and 0–30±2.5 cm soil layer, and (iv) tillage treatments were applied for at least 5 yr (v) soils with clay content between 150 and 350±20 g kg^{-1} was considered.

From each site comparison, the SOC reported at each depth interval was recorded for both NT and CT treatments. Other experiment and site information recorded were mean annual precipitation and temperature, soil texture, duration of tillage study and crops (Table 1). If specific or a number of field experiments, data were collected during more than one period and published separately. Those cases were sometimes difficult to identify but an effort was made to use only the data from the most recent publication. The total data set included 28 studies and contained 76 paired SOC depth measurements (Table 1).

The units for assessing the SOC content varied in the different studies, with some presenting the data as concentration (e.g., grams C per kilogram soil) and others as mass per area for a given depth (e.g., megagrams C per hectare). We used the most general term content for all situations. From each site comparison, the SOC ratio of (NT − CT)/CT was calculated at each depth and for the total C stock from the pair of SOC measurements for each tillage system.

Regression coefficients between the SOC (NT − CT)/CT ratio and MAP and MAT of study were evaluated in the SigmaPlot program.

2.4 Results and Discussion

Information about site locations, length of time, soil and cropping systems of the reviewed studies are presented in Table 1. Soil texture ranged from 15 to 35% of clay content, however, most of the studies were conducted in silt loam soil. Crop rotations varied widely involving annual crops. The duration of the studies ranged from 5 to 44 years, with an average duration of 20 years (Table 1).

In all cases, there was a positive effect of NT on SOC in superficial soil layer (0–5 cm) (Fig. 2). The interactive effects of residue location and the absence of soil disturbance at the soil surface layer contribute to enhance SOC contents at this soil layer (Angers & Eriksen-Hamel, 2008). In addition, laboratory studies have shown that mixing crop residues with soil particles can result in accelerated decomposition relative to residues being left at the soil surface (Coppens et al., 2006).

Table 1. Summary of data for the studies used in the meta-analysis of soil organic C (SOC) under no-till (NT) and conventional tillage (CT).

Reference	Location	Soil texture	Crops	Duration
Venterea et al. (2006)	Minnesota, USA	Silt loam	Maize-soybean	15
Yang & Wander (1999)	Illinois, USA	Silt loam	Maize-soybean	11
Blanco-Moure et al., 2013	Penaflor, Spain	silt loam	Barley	19
Blanco-Moure et al., 2013	Penaflor, Spain	silt loam	Barley-fallow	20
Blanco-Moure et al., 2013	Lanaja, Spain	silt loam	cereal (wheat or barley) - legume	14
Blanco-Moure et al., 2013	Torres de Alcanadre, Spain	sandy loam	cereal (wheat or barley)	9
Blanco-Moure et al., 2013	Undués de Lerda, Spain	silt loam	cereal (wheat or barley) - fallow	13
Blanco-Moure et al., 2013	Artieda, Spain	silt loam	cereal (wheat or barley) - legume	21
Veloso et al. (Submitted-b)	Eldorado do Sul, Brazil	silt loam	maize/winter oat	30
			maize/vetch	30
			maize+cowpea/oat+vetch	
Bayer et al. (2006a)	Luziania, Brazil	Sandy clay loam	Maize-soybean	8
Dimassi et al., 2014	Boigneville, France	Silt loam	maize/winter wheat	41
Hermle et al. (2008)	Tanikon, Switzerzland	Sandy Loam	maize-winter wheat-winter wheat-winter canola	19
Doran et al. (1998)	Nebraska, USA	Silt loam	wheat/fallow	11
Xu et al. (2009)	Loess plateau, China	Silt loam	Spring wheat	7
Deen & Kataki (2003)	Ontariom Canada	Silt loam	Maize/Soybean	25
McConkey et al. (2003)	Saskatechewan, Canada	Fine sand loam	continuous wheat / fallow-wheat	11
McConkey et al. (2003)	Saskatechewan, Canada	Silt loam	continuous wheat / fallow-wheat	12
McConkey et al. (2003)	Saskatechewan, Canada	clay loam	spring wheat/canola	16
Huggins et al. (2007)	Minnesota, USA	clay loam	soybean	14
			maize	14
			soybean/maize	14
Yang & Kay (2001)	Ontario, Canada	Silt loam	countinuous alfafa	20
Dong et al. (2008)	Luancheng, China	Silt loam	winter wheat/maize	5

Continue...

Reference	Location	Soil texture	Crops	Duration
Wang et al. (2008)	Shanxi, China	Silt loam	winter wheat	16
Dieckow et al. (2009b)	Campo Grande, Brazil	Sandy clay	fallow/soybean	11
Poirier et al. (2009)	Québec, Canada	Clay loam	Maize/soybean	13
Jacobs et al. (2010)	Göttingen, Germany	Silt loam	winter wheat-pea-winter wheat/mustard-maize-bean	37
Machado et al. (2006)	Oregon, USA	Silt loam	Winter wheat	22
	Oregon, USA	Silt loam	spring wheat	22
Alhameid et al. (2017)	South Dakota, USA	Fine-silty	Maize/soybean	13
Obour et al. (2017)	Kansas, USA	Silt loam	Wheat-sorghum-fallow	30
Büchi et al. (2017)	Changins, Switzerland	Loam	Wheat/Rapeseed/Maize	44
Kettler et al. (2000)	Nebrasks, USA	Silt loam	Wheat with N /fallow	27
			Wheat without N /fallow	27
Walia et al. (2017)	Illinois, USA	Silt loam	Soybean/Maize	44
Chen et al. (2009)	Chenhuang, China	Silt loam	Winter wheat	11
Xu et al. (2007)	Jiangsu, China	Clay loam	Rice/winter wheat	18
Gwenzi et al. (2008)	Zimbabwe	Sandy loam	Wheat-cotton	5

In superficial soil layer, the difference in SOC accumulation between NT and CT was best fitted in a hyperbolic curve (R^2 = 0.64; p<0.002) as a function of MAP (Fig. 2). The high SOC accumulation in dry sites was likely due to lower SOC mineralization compared to humid sites (Helgason et al., 2014). In addition, the greater soil water content in humid sites compared to drier sites lead to enhance soil organic matter decomposition under NT, particularly in soil surface layer where water content may vary rapidly (Blanco-Moure et al., 2013), minimizing the relative C gain under NT compared to CT (Dimassi et al., 2014). These results are confirmed by many studies that showed greater rate of gain of soil C under no-till than tilled systems in dry regions (less than 600 mm yr^{-1}) (Campbell et al., 2005; Blanco-Moure et al., 2013; Dimassi et al., 2014).

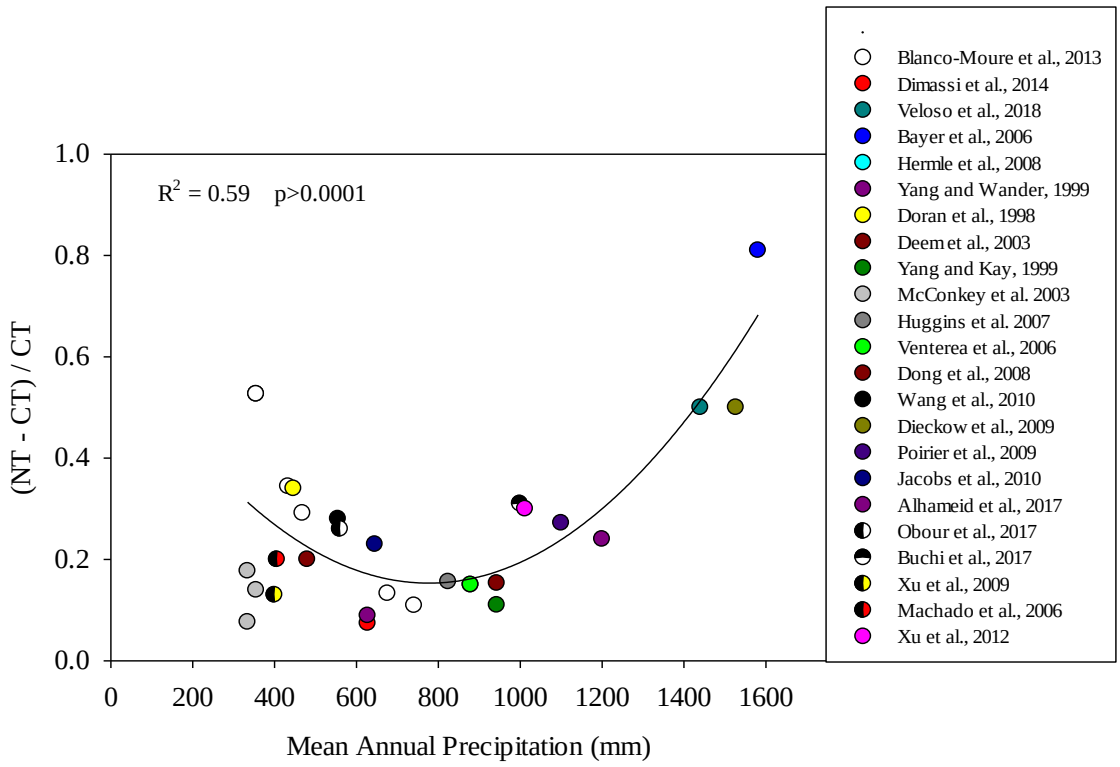

Figure 2. Relative change in soil organic C content under no-till (NT) compared with conventional tillage (CT) in 0–5 cm soil layer as a function of mean annual precipitation.

However, the positive effect on SOC accumulation was stronger at high MAP (> 1000 mm $year^{-1}$), probably due to great capacity of residue input in these sites (Lauenroth et al., 2000; Bai et al., 2008). In this strip of MAP, there was a

great potential to C input through plants which compensated the decomposition of SOM, and consequently favoured the SOC accumulation in the superficial soil layer. The highest gains in SOC accumulation due to NT adoption was observed in tropical and subtropical areas, where the NT resulted in higher soil water content, higher crop yields, and consequently greater soil C storage than CT (Steinbach & Alvarez, 2006).

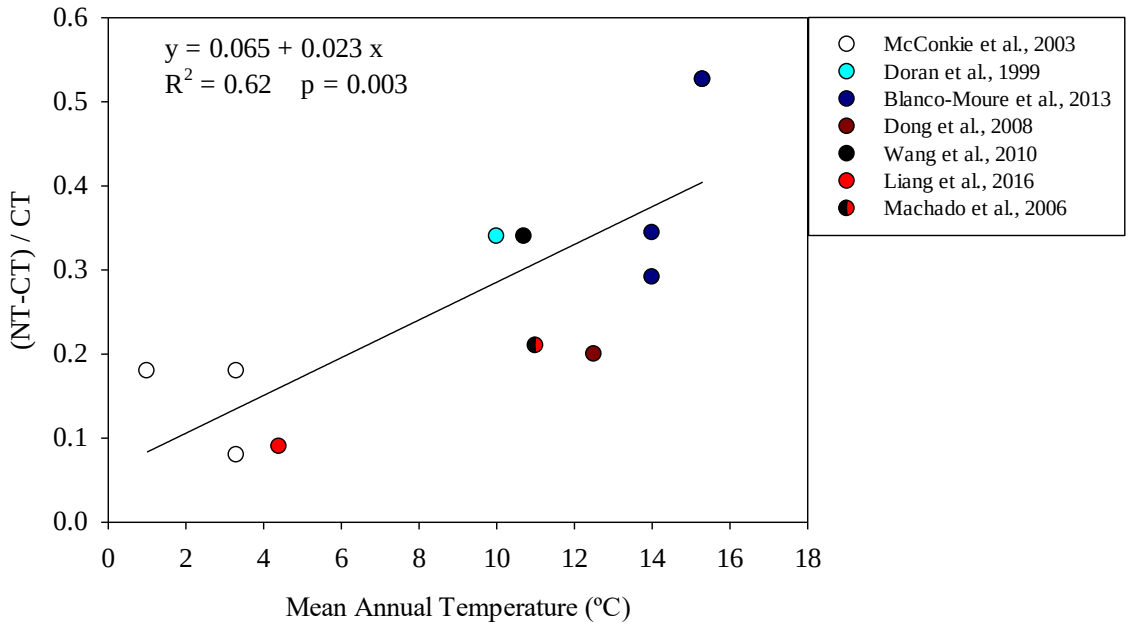

Figure 3. Relative change in soil organic C content under no-till (NT) compared with conventional tillage (CT) in as a function of mean annual temperature in 0–5 cm soil layer of sites with mean annual precipitation < 600 mm.

In 0–30 cm soil layer, the SOC accumulation under NT followed the tendency as the 0–5 cm soil layer, and was still dependent of the MAP (Fig. 4). In sites with MAP below than 1000 mm year^{-1}, the SOC accumulation under NT showed a negative relationship with MAP. This negative relation is possibly due to the mulch effect, that leaving the residues at surface slow down their decomposition (Helgason et al., 2014). Around 500–900 mm year^{-1}, the SOC accumulation under NT was around zero or negative, due possibly to high SOC in deeper soil layers under CT, which have compensation effect with the gains that occurred in surface layer (Angers & Eriksen-Hamel, 2008). In sites with MAP higher than 800 mm year^{-1}, there was still positive effect of NT on SOC, confirming the positive effect of NT at depth with high MAP (Veloso et al.,

Submitted-b). In this study, the authors found that more than 50% of SOC accumulation in whole soil profile was observed in deeper soil layers. The change in the vertical distribution of soil C caused by tillage and cropping systems further highlights the need for deeper soil sampling in soil C study (Angers & Eriksen-Hamel, 2008; Lal, 2009). More studies including C changes in deeper soil layers to a depth of 1m or the depth of crop rooting zone are needed in order to get a full picture of the impact of different cropping and tillage systems (Luo et al., 2010).

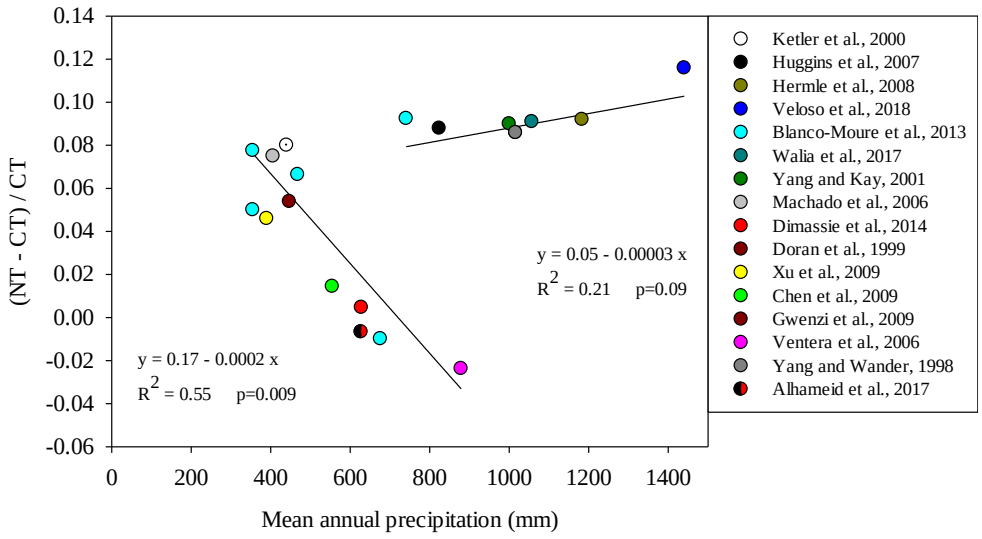

Figure 4. Relative change in soil organic C content under no-till (NT) compared with conventional tillage (CT) in 0–30 cm soil layer as a function of mean annual precipitation.

The MAT also explained the range of SOC accumulation. A positive relationship between MAT and SOC accumulation was observed in sites with less than 600 mm of MAP in the superficial soil layer (Fig. 3). The same tendency was observed in 0–30 cm soil layer (Fig. 5). The positive effect of NT on SOC accumulation under higher temperatures might be explained by the soil covered by straw under NT that favour lower temperature (Grant et al., 1995) and then, lower mineralization intensity of SOC than CT (Alvarez et al., 1995), and consequently favoured the SOC accumulation.

According to a meta-analysis conducted by Virto et al. (2012) differences in SOC stocks between NT and CT were significantly and positively correlated with differences in crop yields. In addition, Pittelkow et al. (2015) found a

relationship for yields comparing NT and CT as function of precipitation: NT performs best relative to conventional tillage under water-limited conditions with tendency of declines in humid sites, yet this was not observed for legumes and oilseeds and cotton crop categories. However, great yields has been observed in sites with high MAP (>1000 mm year^{-1}), as observed in Brazil (Franchini et al., 2012), where NT and crop rotation and NT, allowed high and stable crop yields, especially under water-stress conditions. The author explain the results also relating to the gains in soil organic carbon, that promotes formation and stabilization of soil aggregates, reduction on soil bulk density, higher soil water retention, increases in the cations exchange capacity and nutrient availability, reduction on the activity of toxic mineral elements, such as the aluminum and increases in the amounts, diversity, and activity of the soil biota.

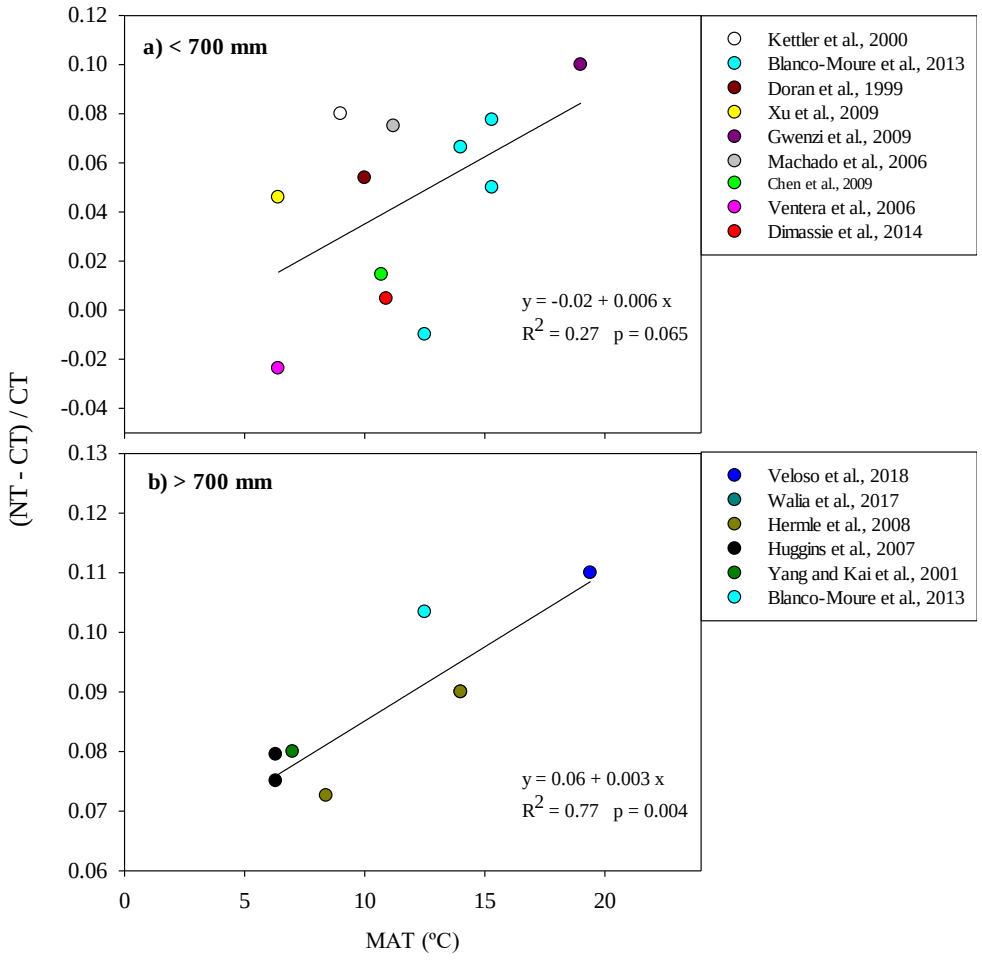

Figure 5. Relative change in soil organic C content under no-till (NT) compared with conventional tillage (CT) in as a function of mean annual temperature in 0–30 cm soil layer of sites with mean annual precipitation < 700 mm (a) and > 700 mm (b).

Thus, our study highlighted that C accumulation under NT is site-dependent because climate condition are controlling effect on the positive balance between input and decomposition of C. One strategy is to adjust crop management practices at a systems-level so that multiple aspects of the tillage system are optimized to improve productivity and environmental outcomes (Haddaway et al., 2016).

2.5 Conclusions

We observed that NT systems are site-specific dependent of climate conditions in order to ensure the C accumulation. Great potential of SOC accumulation under NT compared to CT was observed in regions with MAP less than 500 and more than 1000 mm $year^{-1}$, where likely there is positive balance between input and decomposition of C. The sites with MAP between 600 and 900 mm yr^{-1} showed gains close to zero or negative in 0–30 cm soil layer, due to CT compensate the gains in subsurface layers, indicating that for that regions might favour the SOC accumulation. In addition, this study further highlights the need for deeper soil sampling to get the real impact of tillage systems on SOC.

3. LEGUME COVER CROPS UNDER NO-TILLAGE FAVOR MINERAL-ORGANIC ASSOCIATION IN MICROAGGREGATES AND SOIL C ACCUMULATION[1]

3.1 Abstract

Both no-tillage and legume cover crops have been shown to increase soil organic carbon (SOC) in subtropical soils. However, the mechanisms underpinning management system effects on carbon (C) accumulation are not well understood. We used a combination of aggregate size and density fractionation to elucidate these mechanisms at a long-term site. Two tillage systems (conventional system – CT and no-tillage – NT) combined with three cropping systems [legume oat/maize (O/M), vetch/maize (V/M) and oat+vetch/maize+cowpea (OV/MC)] were evaluated in a 30-year-old experiment carried out on an Acrisol in southern Brazil. Overall, macroaggregation was significantly influenced by tillage with NT showing values 14% greater than CT. Overall, the occluded-C content in macroaggregates was more than twice as high under NT compared to CT (3.8 vs. 1.3 g kg^{-1}). This effect was more pronounced when legume cover crops were grown. However, the most significant effect of cover crops was observed in the mineral-organic fraction of microaggregates under NT (12.1 under O/M and 19.8 g kg^{-1} under OV/MC). Our results suggest that the effect of NT on occluded-C accumulation takes place mainly in macroaggregates through the formation of coarse organic debris leading to mineral-organic associations. However, the practice that had greatest impact on SOC accumulation was the use of legume cover cropping in association with NT which greatly enriched the mineral-associated C in microaggregates.

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[1]Submitted to Soil & Tillage Research

3.2 Introduction

Management systems can make agricultural soils potential sinks or sources of atmospheric CO_2. SOC accumulation is essential for more sustainable and resilient agricultural systems, especially in tropical and subtropical soils, owing to its central role in determining soil properties that strongly affect crop yield and the quality of the wider environment (Lal, 2009; Powlson et al., 2016).

An increase in SOC in agricultural systems can be achieved through management strategies such as reduced tillage or NT (Grandy & Robertson, 2007; Razafimbelo et al., 2008; Fabrizzi et al., 2009). Switching from CT to NT can result in the sequestration of 0.48 Mg ha^{-1} $year^{-1}$ in Brazilian subtropical regions, on average (Bayer et al., 2006a). It has been suggested that the SOC increase found under NT is closely linked to soil macroaggregation. The slower turnover of macroaggregates favors the formation and enrichment of microaggregates within macroaggregates (Six et al., 1999; Denef et al., 2004; Du et al., 2015).

In addition to tillage systems, the use of legume cover crops is an approach that increases nitrogen inputs to the soil, ensuring higher biomass production and SOC accumulation (Diekow et al., 2005b; Amado et al., 2006). In addition to the increase in residue inputs from the cropping systems and the increase in macroaggregation (Conceição et al., 2013), the greater lability of the legume residue may favor mineral-organic interactions (Cotrufo et al., 2013; Cotrufo et al., 2015). The adoption of a consortium of grasses and legumes increases mean weight diameter (MWD) and SOC stocks relative to grass monoculture under NT in subtropical soils in southern Brazil (Conceição et al., 2013). In this same region, Bayer et al. (2009) found SOC sequestration rates varying between 0.11 and 0.68 Mg ha^{-1} yr^{-1}, depending on the cropping system adopted.

Although the impact of agricultural management practices on SOC accumulation has been widely assessed in tropical and subtropical soils, the mechanisms of soil C stabilization affected by different systems are not well understood. Physical fractionation (density or size) has been widely used to study the effect of management systems on SOC stabilization (Diekow et al., 2005a; Alvaro-Fuentes et al., 2009; Andruschkewitsch et al., 2013; Conceição et al.,

2013). There is a wide range of fractionation schemes proposed (Cambardella & Elliott, 1993; Diekow et al., 2005a; Conceição et al., 2008). In general, there are three main fractions: free light fraction (free-POM), occluded light fraction (occluded-POM), and mineral-associated fraction (min-OM) (Conceição et al., 2013). C occlusion and mineral-organic association are considered the main mechanisms of C stabilization in tropical and subtropical soils with a predominance of 1:1 clay and oxides (Conceição et al., 2013; Denef et al., 2007; Zotarelli et al., 2007). Although most of the C is stabilized in the min-OM, Conceição et al. (2013) found that C occlusion in aggregates is equally important, because in addition to physical protection, it provides time for C to be chemically stabilized by minerals.

Therefore, our main objective was to investigate the mechanisms underlying the long-term effect (30-years) of no-tillage and legume cover crops and their effect on C accumulation in a subtropical Acrisol in southern Brazil.

3.3 Materials and methods

3.3.1 Field experiment

This study was based on a long-term field experiment (30 years) in Eldorado do Sul, RS, Brazil. Soil and climate characteristics are presented in Table 2. The experimental area consisted of a native pasture (mainly *Paspalum* spp. and *Andropogon* spp.) and it was converted to traditional system that consisted of conventional tillage (soil was physically degraded) and low levels of crop biomass for approximately two decades. The ongoing experiment, established in 1985, consists of the combination of two tillage [conventional tillage (CT) and no tillage (NT)] systems with three cropping systems [oats (*Avena strigosa* Schreb) / maize (*Zea mays* L.) (O/M), vetch (*Vicia sativa* L.) / maize (V/M) and the consortium of oats + vetch / maize + cowpea (*Vigna ugniculata* (L.) Walp) (OV/MC)] distributed in a randomized block experimental design with split plots and three replicates. Tillage systems were assigned to the main plots (15 × 20 m) and cropping systems to the subplots (5 × 20 m). These tillage systems combined with cropping systems were managed at two levels of fertilization: 0 and 180 kg ha^{-1} of N-urea (0N and

180N), applied in strips in the maize crop, characterized the sub-subplots (5 × 10 m). Only the sub-subplots with no nitrogen fertilization were used in this study.

Table 2. Local climate and soil characteristics of the long-term field experiment (30 years) used in this study, comparing conventional tillage (CT) and no-tillage (NT) which were combined with three cropping systems: (i) black oat (Avena strigosa Schreb) as winter cover crop followed by maize (Zea mays L.) as summer grain crop; (ii) vetch (Vicia sativa) followed by maize and (ii) oat plus vetch followed by maize in summer intercropped with cowpea cover crop (Vigna unguiculata (L.) Walp).

Characteristic		
County	Eldorado do Sul, RS	
Geographic coordinates	30º51'S, 51º38'W	
Altitude (m)	96	
Mean annual temperature (ºC)	19.4	
Mean annual rainfall precipitation (mm)	1440	
Köppen Climate Classification	Cfa	
Soil group (WRB)[1]	Acrisol	
Soil type (US taxonomy)	Typic Paleudult	
Soil texture in 0-20 cm layer	Sandy clay loam	
Particle size distribution		
Clay	220	
Silt	240	
Sand	540	
Mineralogy[2]		
Feo[3]	0.9 g kg^{-1} soil	
Fed[4]	11.8 g kg^{-1} soil	
Feo/Fed	0.08	
Gt/(Gt+Hm)[5]	0.21	
Soil bulk density (Mg m^{-3})[6]	CT	NT
0–5 cm	1.48	1.31
5–10 cm	1.65	1.57
10–20 cm	1.71	1.65

[a] World Reference Base for Soil Resources (IUSS, 2006); [b] Original data presented by Inda-Junior et al. (2007); [c] Ammonium oxalate soluble Fe; [d] Dithionite-citrate-bicarbonate soluble Fe; [e] Gt: goethite; Hm: hematite; [6] NT, no-tillage with glyphosate desiccation prior to maize planting; CT, conventional tillage with plowing and two disking prior to maize planting.

Winter crops were established in April–May in accordance with local technical recommendations. Oats, when cultivated alone, were sown at a rate of 80 kg seed ha^{-1}. When cultivated with vetch, oats were seeded at 30 kg ha^{-1} and vetch at 50 kg ha^{-1}. For vetch cultivated on its own, 80 kg ha^{-1} was used. In the OV/MC system, cowpea (summer cover crop) was sown 15 to 20 days after maize, between the lines of this crop, in pits 40 cm apart, with 3 to 4 seeds per pit. After the maize was harvested, cowpea achieved its full development, remaining until its mechanical management before the winter crops were planted (oats and vetch).

The CT plots were ploughed to a furrow-depth of 17 cm once a year in spring before maize sowing using a three-disk plough and harrowed twice to a depth of 10 cm using a disk harrow mixing the crops residues in this layer. At the same time, glyphosate-based herbicide (Roundup, Monsanto) was applied in the NT plots at 1.4 kg ha^{-1} with respect to final glyphosate concentration, and two or three days later the winter cover crops were managed with a knife-roller and the aboveground residues left on the soil surface. In NT, soil disturbance occurred only in the sowing line, and the residues of the cover crop were left on the soil surface.

Maize was planted with NT sowing in September–October, with 90 cm spacing between rows and using a sowing rate designed to obtain 50–70 thousand plants per hectare. The fertilizer rate applied in maize was 21.5 and 41.5 kg ha^{-1} of P and K, respectively (50 kg ha^{-1} of P_2O_5 and K_2O each). In the treatments with legumes, part of the N supplied to maize occurs through biological fixation.

3.3.2 Soil sampling and soil bulk density and total organic carbon determination

Soil sampling was carried out in September 2014 in the 0−5, 5−10 and 10−20 cm soil layers, prior to soil tillage for maize crop establishment. Trenches were opened to permit the evaluation of the soil bulk density, in duplicate, by the volumetric ring method (volume of 101.5 cm^3) (Blake & Hartge, 1986). Soil samples were collected from the different layers for SOC analysis. The samples were air-dried, ground and passed through a 2-mm-mesh sieve, and then ground in an agate mortar. The determination of SOC concentrations by dry combustion was

performed in the NC soil analyzer (FlashEA 1112, Thermo, Electron Corporation, Milan, Italy).

The SOC stocks up to a depth of 20 cm were calculated using the equivalent mass method (Ellert & Bettany, 1995), using the soil mass of the CT O/M system as reference.

3.3.3 Soil sampling and aggregate size distribution and physical fractionation of SOM

Trenches perpendicular to the sowing lines were excavated with a cutting blade in order to sample undisturbed soil in the 0−5, 5−10 and 10−20 cm layers. These undisturbed samples were manually ruptured at their weakness points until the entire sample could be passed through a 9.51-mm mesh and dried in the shade. After that, aggregates larger and smaller than 2 mm were weighed to determine the proportion of aggregates making up the soil mass required to analyze the aggregate size distribution in water and the physical densimetric fractionation of OM.

To determine the size distribution of aggregates in water, 50 g samples of soil in duplicate were weighed and moistened by capillarity for 12 hours on filter paper. Afterwards, they were transferred to 1000 mL plastic tubes containing 500 ml of water and placed on a rotary shaker for 2 min at 16 rpm. The samples were then transferred to the vertical oscillation shaker, following the procedure of Carpenedo & Mielniczuk (1990). The samples were placed on a set of sieves with mesh sizes 4.76, 2.00, 0.50, 0.25, and 0.053 mm, and shaken in water at 42 oscillations per minute for 15 min, so that water at the lowest level reached the top of the aggregates in the 4.76 mm sieve. The material that passed through the last sieve and was retained in the bucket was flocculated with potassium aluminum (5 g L^{-1}).

The contents of the five sieves and the bottom were transferred to plastic containers of known mass, dried for 48 h at 60 °C and then weighed. The MWD was calculated from the aggregates retained in the five sieves and the bottom (equation 1):

$$MWD = mAGRi \times Ci \times mAGR \quad (1)$$

Where: MWD: mean weight diameter (mm); mAGRi = mass of aggregates class i; Ci = mean value of the class of aggregate obtained [(upper mesh diameter + lower mesh diameter) / 2]; m*AGR* = total mass of aggregates of the six classes.

The aggregates retained in the sieves of 4.76 and 2 mm were pooled, as were classes 0.5 and 0.250 mm and 0.053, and the bottom, making up the large macroaggregates, small macroaggregates and microaggregates, respectively. In these classes, the SOC concentration of was determined by dry combustion in a Thermo elemental analyzer (FlashEA 1112, Thermo, Electron Corporation, Milan, Italy).

For the physical fractionation of SOM from large macroaggregates (> 2 mm), small macroaggregates (2–0.25 mm) and microaggregates (<0.25 mm), 10-g samples from each class of aggregates were placed in centrifuge tubes containing 80 ml of sodium polytungstate solution (SPT) (2.0 g cm^{-3}). After closure of the stopper tube and slow and manual stirring, the free-POM of the OM was released (Conceição et al., 2008). The suspension was centrifuged at 2000 x *g* for 90 minutes. The supernatant, which contained the free-POM, was filtered under vacuum. To remove excess SPT, the free-POM was washed with distilled water and calcium chloride solution ($CaCl_2$) (0.01 mol L^{-1}). The SPT solution (contained in the free-POM supernatant) was returned to the tube and the suspension was subjected to ultrasonic vibration dispersion at the 240 J mL^{-1} level in order to disperse the soil aggregates. This level of energy was previously defined in a specific test and proven to be sufficient to disperse microaggregates > 2 μm in order to obtain 99% of the total clay fraction (Inda Junior et al., 2007).

After sample dispersion, the suspension was centrifuged again and the occluded-POM fraction was separated by following the same procedure as for free-POM. The free-POM and occluded-POM fractions were oven dried at 60 °C, ground in an agate mortar, and analyzed for C using an elemental analyzer (FlashEA 1112, Thermo, Electron Corporation, Milan, Italy). The C-min-OM was

obtained by calculating the difference between SOC and C of the C-free-POM + C-occluded-POM.

3.3.4 Statistical analysis

Data were subjected to analysis of variance (ANOVA) and, when significant ($p < 0.05$), the differences between means of treatments were evaluated by the Tukey test at the 5% level. To compare the effects of the tillage (T) and cropping (CC) systems on the SOC variables, the MIXED procedure was performed, which considered the main factors and their interactions as fixed factors and the block variable and the experimental errors as random variables. All analyses were performed using SAS ® v.9.4 (Statistical Analysis System Institute Cary, North Carolina). The results were interpreted and discussed according to the meaning of the interactions tested or the simple effects, as described in the statistical model.

The statistical model used in the analysis of variance to analyze SOC or C in the classes of aggregates was as follows: $Y_{ijkl} = \mu + B_i + T_j + Error_{(ij)} + C_k + T_jC_k + Error_{(ijk)} + L_l + Error_{(lj)} + T_jL_l + C_k L_l + T_jC_k L_l Error_{(ijkl)}$, where μ = general mean of experiment; B = block (i = 1, 2, 3); T = tillage systems (j = 1, 2); CC = cropping systems (k = 1, 2, 3); L = soil layer (l = 1, 2, 3) e Error = experimental error.

To compare the C concentration in the fractions of SOC within each aggregate class, the statistical model used was follows: $Y_{ijkl} = \mu + B_i + T_j + Error_{(ij)} + C_k + T_jC_k + Error_{(ijk)}$, onde μ = general mean of experiment; B = block (i = 1, 2, 3); T = tillage system (j = 1, 2); C = cropping systems (k = 1, 2, 3) e Error = error experimental.

3.4 Results

3.4.1 Soil organic carbon

The SOC concentration was higher in the superficial soil layer (0–5 cm) under NT compared to CT, but this difference disappeared in the underlying layers (Fig. 6a). The SOC stocks up to a depth of 20 cm depth were similar for the two tillage systems (Table 3). However, the cropping systems had a strong effect on SOC stocks, which varied between 33.1 and 40.8 Mg C ha^{-1}, with a gradual

increase due to legume inclusion in the systems O/M <V/M <OV/MC (Table 4). In addition, the SOC sequestration rate was 0.25 Mg C ha^{-1} yr^{-1} higher in the cropping system with legumes compared to the traditional system with only grasses (O/M) (Table 3).

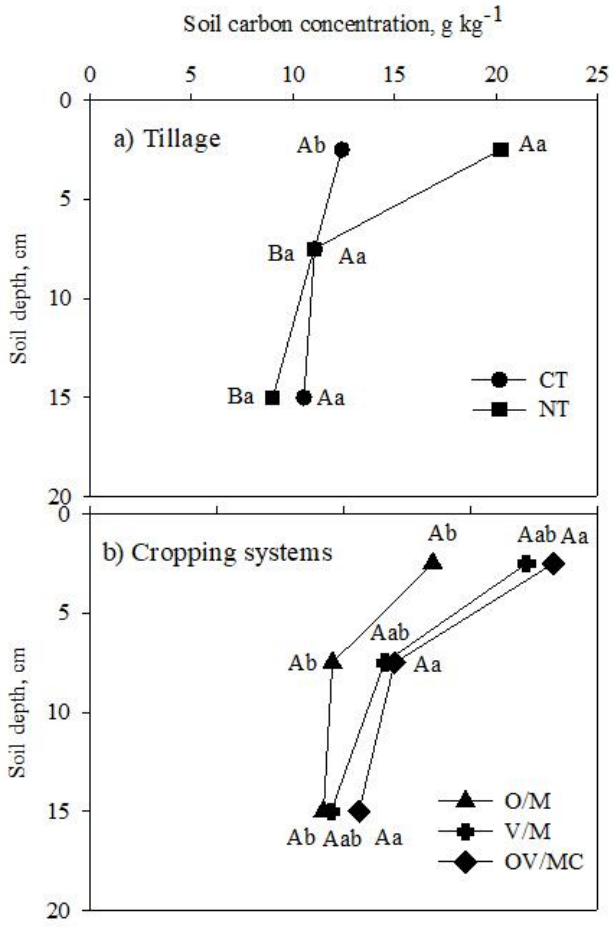


Figure 6. Soil carbon concentration in 0–5, 5–10 and 10–20 cm of sandy clay loam Acrisol subjected to conventional tillage (CT) and no-tillage (NT) (a) in combination with three cropping systems: oat/maize (O/M), vetch/maize (V/M) and oat+vetch/maize+cowpea (OV/MC) (b).

The results were showed in average of cropping systems (a) and of tillage systems (b) because there were not triple interaction. Uppercase letters compare soil layers within the tillage systems (a) or the cropping systems (b). Lowercase letters compare tillage systems (a) or cropping systems (b) within soil layer according to the Tukey test ($p < 0.05$).

Table 3. Soil organic carbon stock in 0–20 cm layer of sandy clay loam Acrisol under conventional tillage (CT) and no-tillage (NT) in combination with three cropping systems: oats/maize (O/M), vetch/maize (V/M) and oat+vetch/maize+cowpea (OV/MC).

Tillage	Crop	SOC stock (Mg ha^{-1})	SOC sequestration (Mg ha^{-1} $year^{-1}$)
CT	O/M	32.4	-
	V/M	37.1	0.16
	OV/MC	39.8	0.25
NT	O/M	33.7	0.05
	V/M	38.0	0.19
	OV/MC	41.7	0.31
Média CT		36.4	0.14
Média NT		37.8	0.18
Média O/M		33.1 b	0.03 b
Média V/M		37.6 a	0.18 a
Média OV/MC		40.8 a	0.28 a
F value	Tillage (T)	0.69 ns	1.00 ns
	Crop (CS)	19.51 **	15.00 **
	T*CS	0.08 ns	0.56 ns

3.4.2 Soil aggregation

The proportions of soil aggregates were strongly affected by tillage systems up to a depth of 20 cm (Fig. 8 a, b, c). In the three soil layers, NT (mean of 34.6%) resulted in a greater proportion of large macroaggregates compared to CT (mean of 20.1%), giving a MWD of 1.3 for CT and 2.2 mm for NT. The cropping systems influenced the proportion of aggregates only in the surface layer. The 40% greater proportion of large macroaggregates under OV/MC was associated with 24% greater MWD compared to O/M (Fig. 8a) in the 0–5 cm depth.

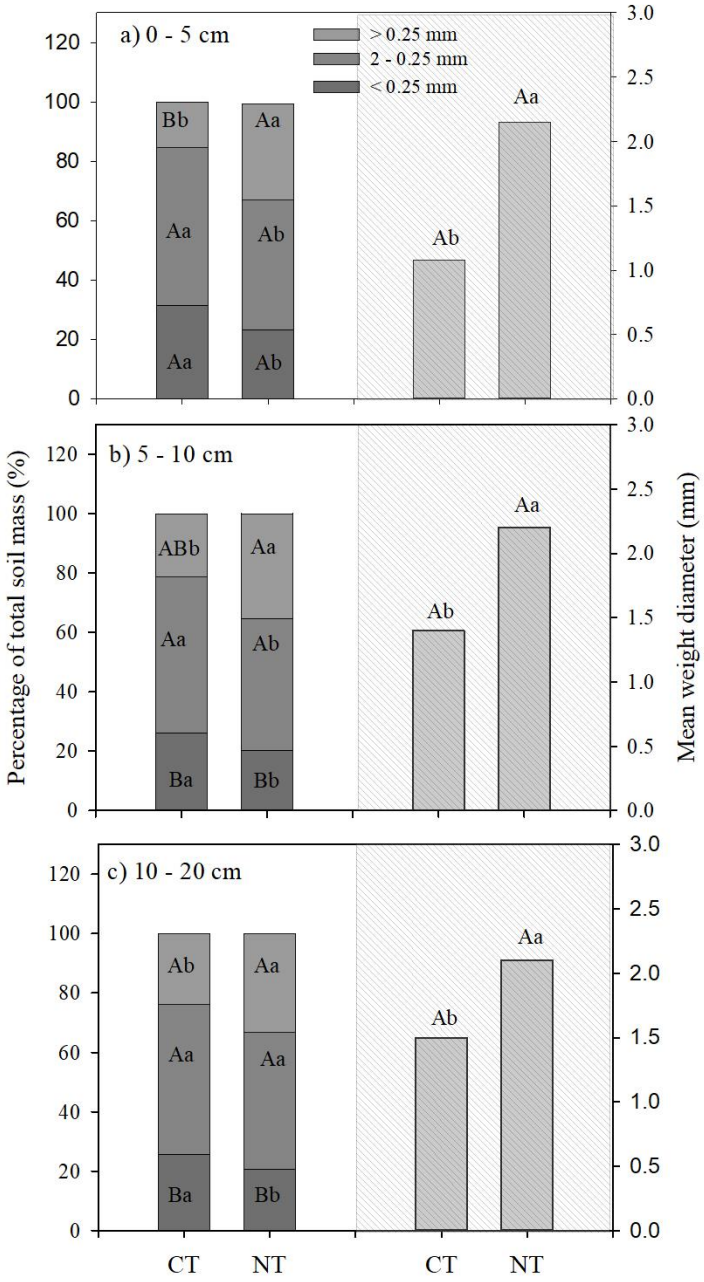


Figure 7. Aggregate size-class distribution and mean weight diameter (MWD) in the (a) 0–5, (b) 5–10 and (c) 10–20 cm layers of a sandy clay loam Acrisol subjected to conventional tillage (CT) or no-tillage (NT).The results were showed in average of cropping systems because there were not triple interaction. Uppercase letters compare soil layers within the tillage systems in each soil aggregate class. Lowercase letters compare tillage systems within soil layer in each soil aggregate class according to Tukey test ($p < 0.05$).

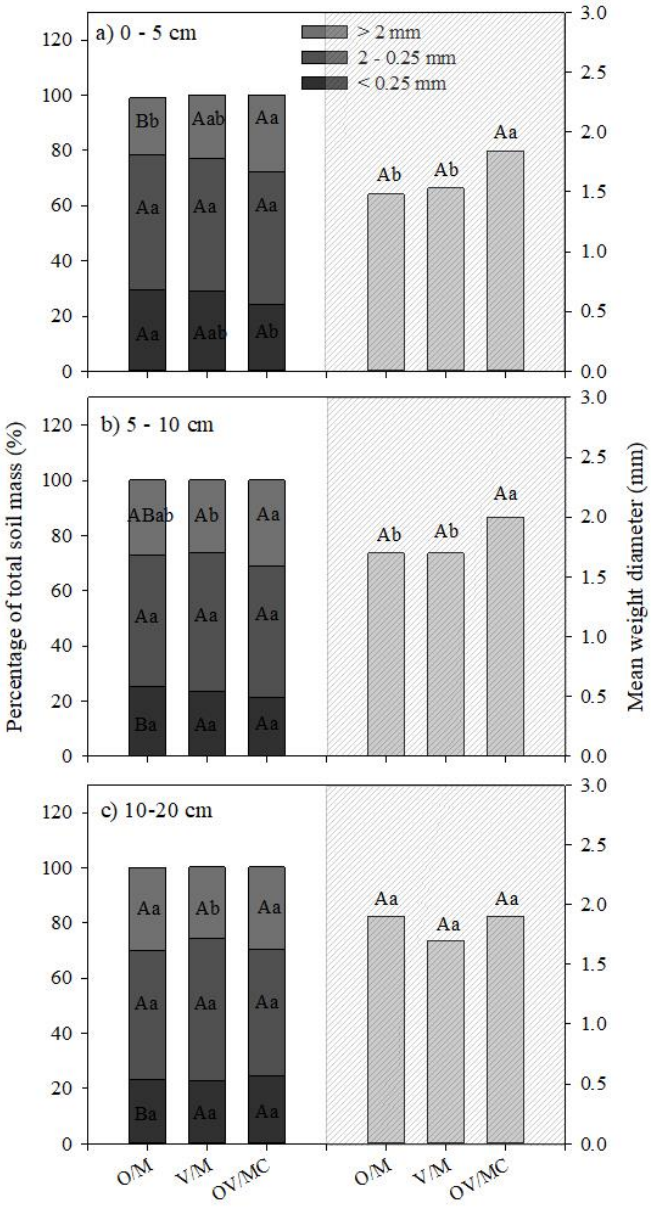


Figure 8. Aggregate size-class distribution and mean weight diameter (MWD) in the (a) 0–5, (b) 5–10 and (c) 10–20 cm layers of a sandy clay loam Acrisol subjected to cropping systems oat/maize (O/M), vetch/maize (V/M) and oat + vetch/maize + cowpea (OV/MC) in average of tillage systems (conventional tillage, CT and no-tillage, NT).The results were showed in average of tillage systems because there were significance only of cropping systems and soil layers. Uppercase letters compare soil layers within the cropping system in each soil aggregate class. Lowercase letters compare cropping systems within soil layer in each class of aggregate according to Tukey test ($p < 0.05$).

3.4.3 SOC concentration in soil aggregates

Tillage systems and cropping systems differed in their effects on SOC concentrations in the three aggregate size classes, mainly in the superficial soil layers (Fig. 9). The SOC concentration in the macroaggregates ranged from 10.27 to 19.46 g kg^{-1}, and was affected mainly by tillage systems (Fig. 9 a, b, c). The increase in SOC concentration in the large and small macroaggregates in the superficial layer under NT was 5 and 4 g C kg^{-1} higher, respectively, than under CT (Fig. 9 a, b). The cropping systems had a greater effect on the small macroaggregates (Fig. 9e) and an especially noticeable effect on the microaggregates in the superficial soil layer (Fig. 9f), with SOC concentrations varying between 15.3 and 21.3 g kg^{-1}. More noticeable, the two legume cover crops (OV/MC) favored C enrichment of 5.4 g kg^{-1} compared to O/M in the microaggregates of superficial soil layer (Fig. 9f).

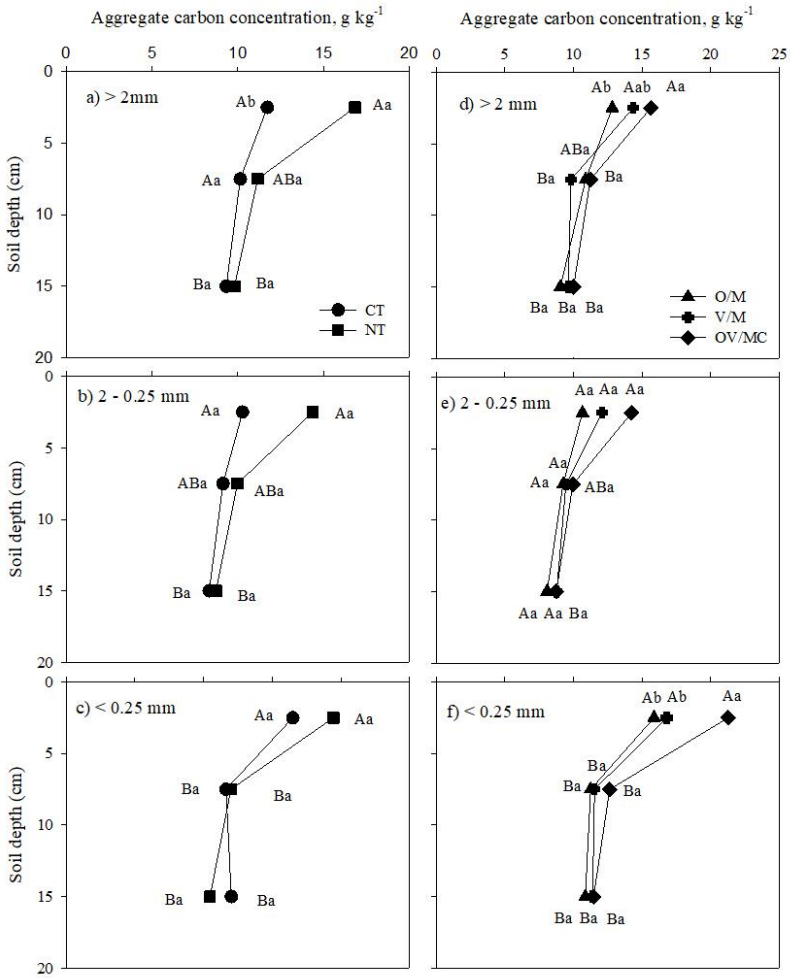


Figure 9. Concentration of organic carbon in aggregates > 2 mm (a, d), 2–0.25 mm (b, e) and < 0.25 mm (c, f) in 0–5, 5–10 and 10–20 cm soil layers of an sandy clay loam Acrisol subjected to conventional tillage (CT) and no-tillage (NT) or to three cropping systems [oat/maize, (O/M), vetch/maize (V/M) and oat+vetch/maize+cowpea (OV/MC). As there were not triple interaction, the results were showed in average of cropping systems because the interaction between tillage system and soil layer (a, b, c) and in average of tillage system because the interaction between cropping system and soil layer (d, e, f). In (a, b, c), uppercase letters compare soil layers within the tillage system in each aggregate class; lowercase letters compare tillage systems within soil layer in each class of aggregate according to Tukey test ($P < 0.05$). In (d, e, f), uppercase letters compare soil layers within the cropping system in each aggregate class; lowercase letters compare cropping systems within soil layer in each class of aggregate according to Tukey test ($p < 0.05$).

3.4.4 Aggregate classes: distribution of physical fractions of C

The C-free-POM fraction of the soil macroaggregates, which represented 9% of the SOC on average, was affected by both tillage and cropping systems (Table 4). This was not the case for the soil microaggregates. On average, the C concentration in the macroaggregates was 2.4 times greater under NT compared to CT. A less pronounced but still important effect was observed for the inclusion of two legume cover crops: the C-free-POM in small macroaggregates was 76% higher with the legume crops compared to O/M.

The C-occluded-POM varied between 1.13 and 4.62 g kg^{-1} in the systems, accounting for between 9.0 and 26.6% of SOC, with a strong influence of soil tillage systems (Table 4). C-occluded-POM was increased 2.2-, 2.8- and 2.6-fold, respectively, in the large and small macroaggregates and the microaggregates under NT compared to CT. The highest C concentration was found in the large macroaggregates under NT (4.93 g kg^{-1}). Despite the less pronounced effect of cropping systems, the C-occluded-POM concentration was increased by 70, 69 and 84% in large and small macroaggregates and microaggregates, respectively, under OV/MC compared to O/M.

Accounting for a major part of the SOC, the min-OM had a higher C concentration compared to C-free-POM and C-occluded-POM. The cropping system had a strong effect on min-OM of microaggregates, essentially under NT (Table 4). The C-min-OM concentration in microaggregates under NT was 64% greater under OV/MC compared to O/M. Although the effect was less pronounced, the main effect of tillage system occurred in macroaggregates, where the C-min-OM concentration was 28% greater in NT compared to CT.

Table 4. Carbon concentration in free particulate organic matter (free-POM), occluded particulate organic matter (occluded-POM) and mineral-associated organic matter (min-OM) in aggregates > 2, 2–0.25 and <0.25 mm of 0–5 cm soil layer of a sandy clay loam Acrisol due to adoption of conventional tillage (CT) and no-tillage (NT) in combination to two cropping systems: oat/maize (O/M) and oat + vetch/maize + cowpea (OV/MC).

Tillage	Crops	> 2 mm			2-0.25 mm			< 0.25 mm		
		Free-POM	Occluded-POM	Min-OM	Free-POM	Occluded-POM	Min-OM	Free-POM	Occluded-POM	Min-OM
		---------- g kg^{-1} fraction ----------								
CT	O/M	1.21	1.13	7.61	0.70	0.99 Ba	7.25	0.48	0.82	14.87 Ba
	OV/MC	1.00	3.28	8.61	1.17	1.67 Ba	7.96	0.41	2.02	14.45 Ba
NT	O/M	1.79	4.14	9.77	2.07	2.80 Ab	7.49	0.70	2.78	12.09 Ab
	OV/MC	1.70	5.72	10.94	3.72	4.74 Aa	9.21	1.20	4.62	19.83 Aa
Mean CT		1.11	2.21	8.11 B	0.94 B	1.33	7.61	0.45	1.42	14.66
Mean NT		1.75	4.93	10.36 A	2.90 A	3.77	8.35	0.95	3.70	15.96
Mean O/M		1.50	2.64	8.69	1.39 b	1.90	7.37	0.59	1.80	13.48
Mean OV/MC		1.35	4.50	9.78	2.45 a	3.21	8.59	0.81	3.32	17.14
F	Tillage	4.58 ns	9.05 ns	21.64 *	26.80 *	223.35 **	0.96 ns	2.60 ns	3.95 ns	0.61 ns
	Crops	0.94 ns	3.11 ns	0.01 ns	7.86 *	175.66 **	3.96 ns	1.44 ns	5.57 ns	23.60 **
	T*CS	0.22 ns	0.23 ns	1.74 ns	2.45 ns	66.76 **	0.70 ns	2.14 ns	0.76 ns	29.34 **

No significant differences ($p > 0.05$) between treatments are designated with (^{ns}). Significant differences between treatments at 5% and 1% probability of error by test F are designed with (*) and (**), respectively. Uppercase letters compare tillage systems within cropping systems and lowercase letters compare cropping systems within tillage systems.

3.5 Discussion

The tillage systems had a significant influence on soil aggregates, especially macroaggregates, with NT showing values 14% greater than NT. These macoaggregates under NT were also enriched in occluded C. Macroaggregates are complex structures, composed of labile organic material recently added to the soil (Angers & Giroux, 1996; Six et al., 1999; Puget et al., 2000; Plaza-Bonilla et al., 2013). Consequently, the disruption of macroaggregates in CT systems can reduce C occlusion, releasing protected organic material and contributing to its decomposition (Andruschkewitsch et al., 2013; Andruschkewitsch et al., 2014; Cates et al., 2016). In contrast, soil macroaggregates made a strong contribution to C occlusion in NT systems (Table 4), where they accounted for approximately 80% of this fraction. The C-occluded-POM in macroaggregates explain the difference between NT and CT in the level of C stocks in the superficial soil layer. The higher proportion of macroaggregates in NT compared to CT contributed to higher C stocks from the C-occluded-POM (2.38 vs. 0.77 Mg ha^{-1}, on average, data not shown).

The greater C occlusion in macroaggregates in NT soil compared to CT favored the formation of mineral-organic associations (Figure 10a, b). This may be because aggregates “provide time” for C-occluded-POM to become associated with the mineral soil matrix forming an mineral-organic association (Conceição et al., 2013; Garcia-Franco et al., 2015). When the C-min-OM stock in large macroaggregates of superficial soil layer is calculated, the difference between NT and CT amounts to 2.46 and 0.92 Mg ha^{-1} (data not shown), respectively. These results point to reduced aggregate turnover under NT relative to CT (Six et al., 1999) favoring the physical protection of particulate OM as well as interactions between soil mineral particles and OM in the superficial soil layer (Courtier-Murias et al., 2013).

However, this difference in carbon accumulation in macroaggregates between tillage systems was not apparent in the 5–20 cm soil layer; hence, carbon stocks in the 0–20 cm layer were similar (Table 3). In the 5–20 cm soil depth, the SOC content under NT was similar to that of the CT treatment (Fig. 6). Plaza-Bonilla et al. (2010) and Andruschkewitsch et al. (2014) likewise found that the higher SOC content within macroaggregates under the NT treatment was restricted to the 0–5 cm soil depth.

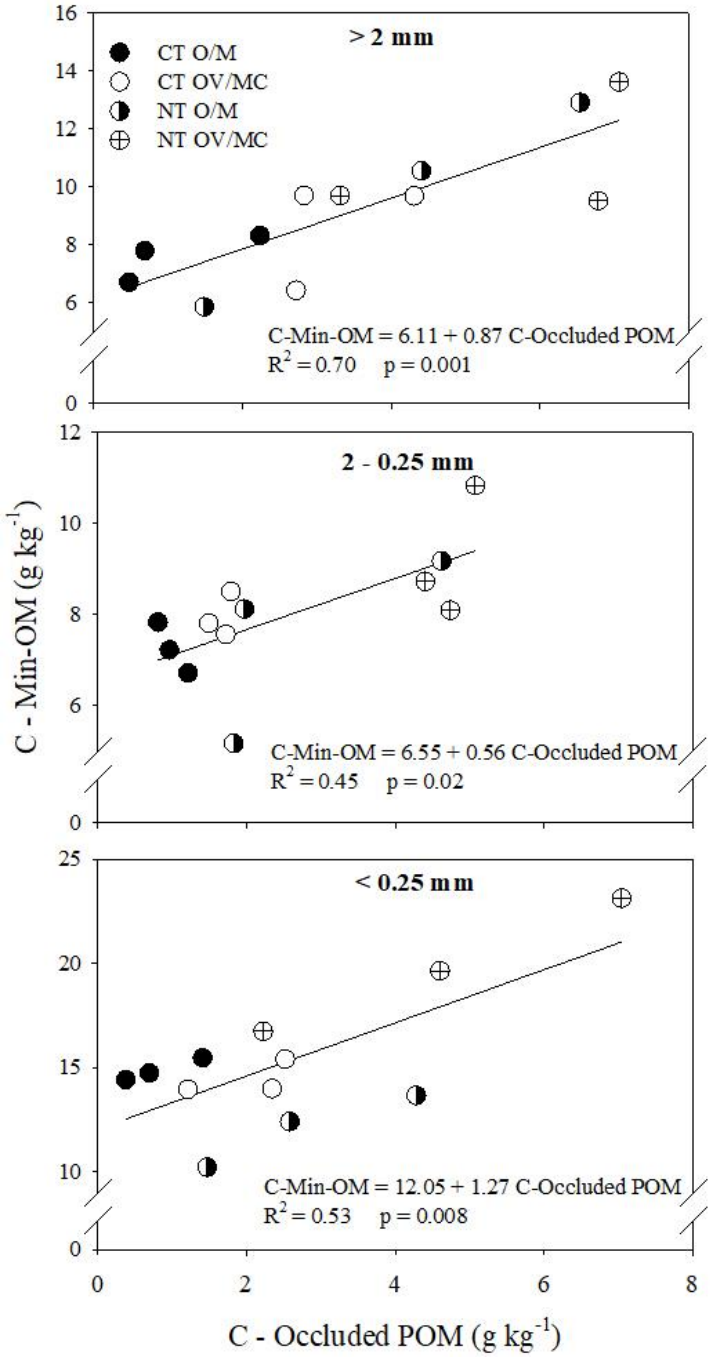


Figure 10. Regression of C concentration in the mineral associated organic matter (C-Min- MO) as a function of the concentration of C in the occluded particulate organic matter (C-FLO) in aggregates > 2 (a), 2–0.25 (b) and <0.25 mm (c) in 0–5 cm layer of sandy clay loam Acrisol under conventional tillage (CT) and no-tillage (NT) in combination with oat/corn (O/M) and oat+vetch /maize+cowpea (OV/MC).

The strongest effect on mineral-organic association occurred mainly as a result of the influence of cover crops in soil microaggregates, particularly in the NT system. When calculated, the C-min-OM stock in microaggregates under NT (data not shown) was 3.0 vs. 2.0 Mg ha^{-1} for OV/MC and O/M, respectively. The greater lability of the legume cover crop residues, together with the greater residue input, contributed to more efficient use of this material by microorganisms. The resulting microbial residue is stabilized by chemical bonding with the mineral soil matrix (Cotrufo et al. 2013; Cotrufo et al., 2015).

Overall, the C-min-OM fraction accounted for up to 75% of the SOC and was also the fraction most influenced by cropping systems (Table 4). The cropping system was the main factor that affected SOC accumulation up to 20 cm depth due to great capacity to affect the C-min-OM. The greater amount and lability of the residue inputs associated with the use of two legume cover crops (OV/MC) played a key role in increasing SOC accumulation by 0.25 Mg ha^{-1} $year^{-1}$ in relation to the O/M system. Although NT increased the C-occluded-POM compared to CT, this effect was smaller than the benefits that legume cover crops can offer in terms of gains in mineral-organic association.

3.6 Conclusions

The mechanisms by which no-tillage and legume cover crops favor soil C accumulation in a subtropical Acrisol differ. While no-tillage increases C occlusion, legume cover crops are associated with an increase in the amount and lability of residue inputs, which favor mineral-organic associations in fine particles of soil, leading to C accumulation to a soil depth of 20 cm. Accordingly, legumes crops have major impact on long-term SOC stabilization and SOC accumulation.

4.THE MEANINGFUL BUT DIFFERENTIAL ROLE OF NO-TILLAGE AND LEGUME COVER CROPS IN STABILIZING SOIL CARBON

4.1 Abstract

No-tillage coupled with legume cover crops usually result in large increase of organic carbon (C) in tropical and subtropical soils, but stabilization mechanisms at work remain largely unknown. A 6-month incubation experiment using intact and crushed soil aggregates was performed to assess the role of physical protection and mineral-organic association on the mean residence time of C (MRT) in a subtropical Acrisol. The study was based on soil samples collected in a long-term (30 years) field experiment involving two tillage (CT-conventional tillage and NT-no tillage) and two cropping systems (O/M- black oat/maize and OV/MC – black oat+vetch/maize+cowpea). Using a double exponential decay model, we estimated the fast and slow soil C pools, oxidation rates and the MRT of C in large (>2 mm) and small macroaggregates (0.25–2 mm), and microaggregates (<0.25 mm). A large difference was observed in the MRT of the fast (< 0.13 years) and slow pools (23 to 297 years). The oxidation rate of C was about 10 times higher in macro than microaggregates. The MRT of soil microaggregates under NT coupled with legume cover crops was 297 years which is 18% higher than under cereal-based cropping system. By the difference in intact and crushed soil aggregates, the results suggest that occlusion was the main mechanism driving C stabilization in large and small soil macroaggregates, responsible for more than 70% of the MRT under NT OV/MC. On the other hand, the mineral-organic association favoured by legume cover crops under NT was the main driver of C stabilization in microaggregates, contributing to 65% of the MRT. Our results highlight the meaningful and differential roles of no-tillage and legume cover crops on C stabilization with the former favoring occlusion in soil macroaggregates and the latter, mineral-organic association in soil microaggregates. Overall, our results provide field-based evidence for a particular role of high-quality plant residues (legume cover crops) in stabilizing soil carbon.

4.2 Introduction

Long-term C stabilization in soil is crucial to soil and environmental quality (Lal, 2004a, 2015). In humid subtropical and tropical climates, high solar radiation and humidity during all year result in high rates of organic matter oxidation and nutrient cycling in agricultural soils, compared to temperate environments (Balesdent et al., 1990; Bayer et al., 2006b). Conservation management systems involving no-tillage (NT) and high C input cropping systems usually result in large increase of organic carbon (C) in tropical and subtropical soils (Diekow et al., 2005a; Bayer et al., 2006b; Conceição et al., 2013).

Both physical protection and mineral-organic associations are the main mechanisms of C stabilization in tropical and subtropical soils (Dieckow et al., 2009a; Conceição et al., 2013). Studies show that occlusion of C in aggregates or in association with soil minerals increases its MRT up to four times, reaching thousands years, compared to biochemically protected C (<50 yr) (Marschner et al., 2008). By increasing the occlusion of C in large soil macroaggregates, NT favours C enrichment in microaggregates (Six et al., 1999; Plaza-Bonilla et al., 2013). The same mechanisms can also be triggered by legume cover crop, mainly by accrual C in mineral-organic association (Veloso et al., Submitted-a).

In addition, soil aggregate size classes represent distinct pools of soil C (von Lutzow et al., 2006; Yamashita et al., 2006; Rabbi et al., 2015), with younger and more labile organic compounds in soil macroaggregates (> 0.25 mm) and more persistent fractions in soil microaggregates (<0.25 mm) (Cambardella & Elliott, 1993; Angers & Giroux, 1996; Puget et al., 2000; Six et al., 2000). Consequently, greater MRT was found in soil fine fractions compared to macroaggregates (Monreal et al., 1997).

There are still few studies looking specifically at the contribution of C stabilization mechanisms on the residence time of C in soils. The hypothesis of this study was that the absence of soil disturbance combined with legume cover cropping increase physical protection of organic matter in macroaggregates and mineral-organic associations in microaggregates, resulting in increasing their MRT. We assessed these mechanisms using a 6-month incubation of intact and crushed soil aggregates from a long-term field experiment in a subtropical Acrisol in Southern Brazil.

4.3 Materials and Methods

4.3.1 Description of field experiment

This study was based on soil samples collected from an on-going 30 years-old experiment conducted in Eldorado do Sul, RS, Southern Brazil. The climate region is Cfa (Köppen), with average temperature and annual rainfall of 19.4 °C and 1440 mm, respectively. The soil is classified as a sandy clay loam granite-derived Acrisol (FAO, 2015), with 220 g kg^{-1} clay in the 0 to 20 cm superficial layer. More detailed soil and climate characteristics are presented in Table 5.

The experiment, established in 1985, consists of the combination of three tillage [conventional tillage (CT), reduced tillage (RT) and no tillage (NT)] in the main plots (15 × 20 m) and three cropping systems [oat (*Avena strigosa* Schreb)/maize (*Zea mays* L.) (O/M), vetch (*Vicia sativa*)/maize (V/M) and the consortium of oat+vetch/maize+ cowpea (*Vigna ugniculata* (L.) Walp) (OV/MC)] in the split-plots (5 × 20 cm) distributed according to a randomized block experimental design, with three replicates. These tillage systems combined with cropping systems were managed at two levels of fertilization, 0 and 180 kg ha^{-1} of N-urea (0N and 180N), applied in strips in the maize crop. However, for the purpose of this study, only extreme soil tillage (CT and NT) and cropping systems (O/M and OV/MC), without N fertilization, were selected.

Winter crops were sown at fall season (April-May in the South Hemisphere) following local technical recommendations. Oat, when cultivated alone, was sowed with a seed rate of 80 kg ha^{-1}. For the oat+vetch consortium, the seeding rates were 30 and 50 kg ha^{-1} for oat and vetch, respectively. The maize was sowed in spring (September-October) spaced 90 cm between rows and plant population of 50–70 thousand per hectare. The cowpea was sown 20 to 30 days after maize, between the lines of this crop, in pits distant 40 cm apart. After harvesting the maize, the cowpea remained until its mechanical management before the seeding of the winter crops (oats and vetch).

Table 5. Climate and soil characteristics of the long-term field experiment (30 years) used in this study, comparing conventional tillage (CT) and no-tillage (NT) combined with three cropping systems: (i) black oat (Avena strigosa Schreb) as winter cover crop followed by maize (Zea mays L.) as summer grain crop (O/M) and (ii) oat plus vetch (Vicia sativa) followed by maize in summer intercropped with cowpea cover crop (Vigna unguiculata (L.) Walp) (OV/MC).

Characteristic					
Soil group (WRB)[1]			Acrisol		
Soil type (US taxonomy)			Typic Paleudult		
Soil texture in 0–20 cm layer			Sandy clay loam		
Mineralogy[2] (g kg^{-1} solo)					
Feo[3]			0.9		
Fed[4]			11.8		
Feo/Fed			0.08		
Gt/(Gt+Hm)[5]			0.21		
		CT		NT	
Soil carbon (g kg^{-1})[6]		O/M	OV/MC	O/M	OV/MC
0–5 cm		10.9	13.1	16.1	23.4
5–10 cm		9.7	11.5	9.4	12.5
		CT		NT	
Aggregate size distribution (%)[6]		O/M	OV/MC	O/M	OV/MC
0–5 cm	> 2 mm	12.5	18.6	29.2	37.3
	2 – 0.25 mm	52.6	53.9	45.1	42.3
	< 0.25 mm	34.9	27.5	23.7	20.4
		CT		NT	
Occluded-POM-C (g kg^{-1})[6,7]		O/M	OV/MC	O/M	OV/MC
0–5 cm	> 2 mm	1.13	3.28	4.14	5.72
	2 – 0.25 mm	0.99	1.67	2.80	4.74
	< 0.25 mm	0.82	2.02	2.78	4.62
		CT		NT	
Min-OM-C (g kg^{-1})[6,8]		O/M	OV/MC	O/M	OV/MC
0–5 cm	> 2 mm	7.61	8.61	9.77	10.94
	2 – 0.25 mm	7.25	7.96	7.49	9.21
	< 0.25 mm	14.87	14.45	12.09	19.83

[1] World Reference Base for Soil Resources (IUSS, 2006). [2] Original data presented by Inda-Junior et al. (2007). [3] Ammonium oxalate soluble Fe. [4] Dithionite-citrate-bicarbonate soluble Fe. [5] Gt: goethite; Hm: hematite. [6] Original data presented by Veloso et al. (Submitted-a). [7] carbon content in occluded particulate organic matter (occluded-POM). [8] carbon content in mineral-associated organic matter (min-OM).

Tillage operations were performed only once a year preceding maize planting (spring). In CT system, the residue was incorporated by plowing once (17 cm depth) and two disking operations (10 cm depth). In NT, sowing was performed through the crop residues of the previous crop.

Annual carbon addition by cropping systems (shoot and root, considered root 30% of the shoot) was calculated using a database with information from the beginning of the experiment to 2005 (Zanatta et al. 2007), and updated until the year 2014. This study presents the C addition to O/M (4.99 Mg C ha^{-1} $year^{-1}$) and OV/MC (7.32 Mg C ha^{-1} $year^{-1}$) in average for CT and NT systems, which presented the same biomass production for the whole experimental period.

4.3.2 Soil sampling and aggregate size distribution and physical fractionation of SOM

Undisturbed soil samples from the 0−5 and 5−10 cm soil layers were collected in manually open trenches. The soil samples were manually ruptured at their weakness points until the entire sample passed in a 9.51 mm mesh and dried at shade. The proportion of aggregates larger and smaller than 2 mm was then assessed and was used to compose the representative soil samples for evaluation of the aggregate size distribution in water and the physical densimetric fractionation of organic matter.

The aggregate size distribution in water was performed according to Carpenedo & Mielniczuck (1990). Briefly, approximately 50 g soil, in duplicate, were moistened by capillarity for 12 hours on a filter paper. Afterwards, soil was transferred to 1000 mL plastic tubes containing 500 ml of water and placed on a rotary shaker for 2 minutes at 16 rpm, with the objective to do a standardized pre-breakdown because aggregates of subtropical and tropical soils are very stable, in general (Carpenedo & Mielniczuk, 1990; da Silva & Mielniczuk, 1997). Soil aggregates were placed on a stack of sieves with mesh sizes of 4.76, 2.00, 0.50, 0.25, and 0.053 mm, shaken in water at 42 vertical strokes per minute for 15 minutes, ensuring that water reached the surface of the aggregates in the 4.76 mm sieve. The soil that passed in the last sieve was retained in the bucket and flocculated with potassium alum ($KAl(SO_4)_2$, 5 g L^{-1}). The soil remaining in the five sieves plus the soil <0.053 mm was transferred into plastic jars, dried for 48 h at 60 °C and weighed. The aggregates from the different size classes were

pooled to compose the following classes: large macroaggregates (>4.76 mm and 2–4.76 mm), small macroaggregates (0.5–2.0 mm and >0.250 mm) and microaggregates (0.053–0.250 mm and <0.053 mm).

The densimetric fractionation of organic matter was performed by weighing twenty grams of soil in centrifuge tubes containing 80 mL of solution of sodium polytungstate (PTS) (2.0 g cm^{-3}). After closure of the stopper tube and slow and manual stirring, the free-POM was released (Conceição et al., 2008). The suspension was centrifuged at 2000g for 90 minutes, and the supernatant, containing the free-POM, was filtered under vacuum. To remove excess PTS, the free-POM was washed with distilled water and a 0.01 mol L^{-1} $CaCl_2$ solution. The remaining PTS solution (containing occluded+mineral associated organic matter) was subjected to ultrasonic vibration dispersion at the 240 J mL^{-1} level in order to disperse the soil aggregates. This level of energy was previously defined in a specific test and was proven to be sufficient to obtain a 99% dispersion of the total clay fraction (Inda Junior et al., 2007). After soil dispersion, the suspension was centrifuged again and the occluded-POM fraction separated following the same procedure as for free-POM. The free-POM and occluded-POM fractions were oven dried at 60 °C, ground in an agate mortar, and analyzed for C by elemental analyzer. The C-min-OM (mineral-associated organic matter) was obtained by the difference between total soil organic C (SOC) and the C of POM fractions (free-POM + occluded-POM).

4.3.3 Estimation of carbon pools, oxidation rates e mean residence time

Intact and crushed samples of large and small macroaggregates, and microaggregates from the surface soil layer (0–5 cm) were subjected to a 6-month incubation. Briefly, 4 g of intact and crushed aggregates were placed in 23 mL flasks with caps and incubated at 25 °C and at 40% moisture content (w/w). The flasks were opened for fifteen minutes to aerate and closed for an hour. Air samples from inside the flasks were collected with 20 mL polypropylene syringes. For the time zero, during the bottle closure, the atmosphere of the laboratory was sampled.

The CO_2 concentration in air samples was analyzed with a Shimadzu GC 2014. With the gas concentration and headspace volume, the rate of CO_2 production (g h^{-1} per kg soil aggregate) was calculated according to Equation 1.

$$f = \frac{\Delta Q}{\Delta t} \frac{PV}{RT} \frac{M}{Msoil}$$

equation 1

where *f* is the CO_2 production rate (g h^{-1} per kg soil), $\Delta Q/\Delta t$ the change in gas concentration (mol h^{-1}) in the headspace, *P* the atmospheric pressure in the flask (assumed as 1 atm), *V* the headspace volume (L), R the ideal gas constant (0.0825 atm L mol^{-1} K^{-1}), *T* the temperature (K) inside of flask, *M* the gas molar mass (44 g mol^{-1}) and *Msoil* the mass of soil aggregate (kg).

Total CO_2 evolved during the 6-month period (30 January to 21 July of 2015) was calculated for intact and crushed soil aggregate samples, by integrating the CO_2 release rate (g h^{-1} per kg soil aggregate) using trapezoidal interpolation.

Based on CO_2 release of the soil aggregate classes, we estimated parameters of C dynamics in the soil aggregates, such as the size of the fast and slow pools (% of total C), oxidation rate ($year^{-1}$), and the mean residence time (years). The soil C losses, calculated as the difference between the SOC content of the sample and the total C-CO_2 released along the incubation period, were fitted to a double exponential decay model (fast and slow pools) (Knicker et al., 2013) using the SigmaPlot 12.0 program (Systat software, Inc.) according to equation 2:

$$A(t) = (Pool_{fast} \times e^{-k2ft}) + (Pool_{slow} \times e^{-k2st})$$

equation 2

where, A(t) = C remaining (% of SOC); $Pool_{fast}$ = amount of C, which is relatively labile, but not mineralized (% of SOC); $Pool_{slow}$ = amount of C, which is more stable but not mineralized (% of SOC); t = time of incubation; k_2f e k_2s = constants of first-order mineralization rates for fast and slow pools ($year^{-1}$), respectively. Therefore, considering first order kinetics, the mean residence time of the two C-pools (MRT_{fast} and MRT_{slow}) were calculated as $1/k_2$f and $1/k_2$s.

We considered physical protection and mineral-organic association as the dominant mechanisms controlling C stabilization of slow pool (Conceicao et al., 2013). Therefore, we assumed that the sum of physical protection and mineral-organic association contributions accounted for 100% of MRT_{slow}. The contribution of physical protection to the MRT_{slow} was calculated as the difference between MRT_{slow} of crushed and intact samples, based on the additional CO_2 released from crushed than intact soil samples. The contribution of mineral-organic association to the MRT_{slow} was calculated as the difference between intact soil samples and the contribution of physical protection.

4.3.4 Statistical analysis

The results were first analyzed for normality using the Kolmogorov-Smirnov test and homogeneity of variance with the Levene test. Results were then submitted to analysis of variance (ANOVA) and, when significant ($p<0.05$), the difference between means were evaluated using the Tukey's test ($p <0.05$).

The statistical analysis was performed using SAS ® v.9.4 (Statistical Analysis System Institute Cary, North Carolina). The MIXED procedure was performed to compare the effects of the tillage (T) and cropping systems (CS) on the response variables, considering the main factors and their interactions as fixed factors and the block variable and the experimental errors as random variables.

The statistical model used in analysis of variance to analyze soil organic matter fraction was: $Y_{ijkl} = \mu + B_i + T_j + Error_{(ij)} + C_k + T_jC_k + Erro_{(ijk)} + L_l + Error_{(lj)} + T_jL_l + C_k L_l + T_jC_k L_l Error_{(ijkl)}$, where μ = general mean of experiment; B = block ($i = 1, 2, 3$); T = tillage systems ($j = 1, 2$); CC = cropping systems ($k = 1, 2, 3$); L = soil layer ($l = 1, 2, 3$) and Error = experimental error.

Regression analyses were performed (SigmaPlot for Windows v. 13.0, Systat Software, Inc., San Jose, CA) to explore the relationships between the additional CO_2 from crushed soil aggregates and occluded-POM-C (from Veloso et al., submitted-a) and also between the MRT_{slow} of the soil aggregates and Min-OM-C (from Veloso et al., submitted-a).

4.4 Results

4.4.1 Soil organic carbon fractionation

The content of free-POM-C ranged from 0.74 to 3.16 g C kg^{-1} in the superficial soil layer and was three times greater under NT than CT. In the superficial layer under NT, the content of free-POM-C was 89% greater under OV/MC than O/M (Table 6). The occluded-POM-C was strongly influenced by the different systems in the surface soil layer, ranging from 1.49 to 4.08 g C kg^{-1} soil. Occluded POM-C was 69% greater under NT than CT and 84% greater under OV/MC compared to O/M.

Table 6. Carbon content in free particulate organic matter (free-POM), occluded particulate organic matter (occluded-POM) and mineral-associated organic matter (min-OM) in 0–5 and 5–10 cm layer of Acrisol subjected to conventional tillage (CT) and no-tillage (NT) in combination with two cropping systems: oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC).

Tillage	Crops	C content (g kg^{-1})			
		Free-POM	Occluded-POM	Min-OM	Total
			0–5 cm		
CT	O/M	0.74 Ba*a*	1.49 Ba*b*	8.33 Ba*b*	10.56 Ba*b*
	OV/MC	0.78 Ba*a*	2.17 Ba*a*	10.43 Ba*a*	13.38 Ba*a*
NT	O/M	1.67 Aa*a*	2.03 Aa*b*	11.27 Aa*b*	14.97 Aa*b*
	OV/MC	3.16 Aa*a*	4.08 Aa*a*	13.62 Aa*a*	20.86 Aa*a*
			5–10 cm		
CT	O/M	0.46 Ab*a*	1.40 Aa*a*	7.60 Aa*b*	9.46 Aa*b*
	OV/MC	0.30 Ab*a*	1.46 Ab*a*	9.98 Aa*a*	11.74 Aa*a*
NT	O/M	0.36 Ab*a*	1.39 Ab*a*	7.19 Ab*b*	8.94 Ab*b*
	OV/MC	0.24 Ab*a*	1.66 Ab*a*	9.78 Ab*a*	11.68 Ab*a*

Uppercase letters compare tillage systems within soil layers and crop systems. Lowercase letters compare soil layers within tillage and cropping systems. Italic lowercases letters compare cropping systems within soil layers and tillage systems according to Tukey test ($p < 0.05$).

The C content in Min-OM-C ranged from 8.3 to 13.6 g $kg^{-1}$of whole soil and represented more than 62% of the total SOC in 0–5 cm layer (Table 6). Min-OM-C was 32% greater under NT than CT and 23% under OV/MC than O/M. At 5–10 cm layer, the min-OM-C was the predominant OM fraction. However, in this soil layer, unlike the tillage system that did not influence the accumulation of min-OM-C, the content of Min-OM-C was 34% greater under OV/MC compared to O/M.

4.4.2 Cumulative loss of C

The Supplementary 1 shows the loss of C in intact and crushed samples of large macroaggregates, small macroaggregates and microaggregates as a function of incubation time. All mineralization curves showed very good coefficients of determination (R^2> 0.97). In six months of incubation, total C losses were less than 3.5%, with lower values in microaggregates (Supplementary 1c, f). Macro (average of large and small) and microaggregates showed a mean C loss of 0.38 and 0.21g kg^{-1}, respectively (Fig. 11 a,b,c).

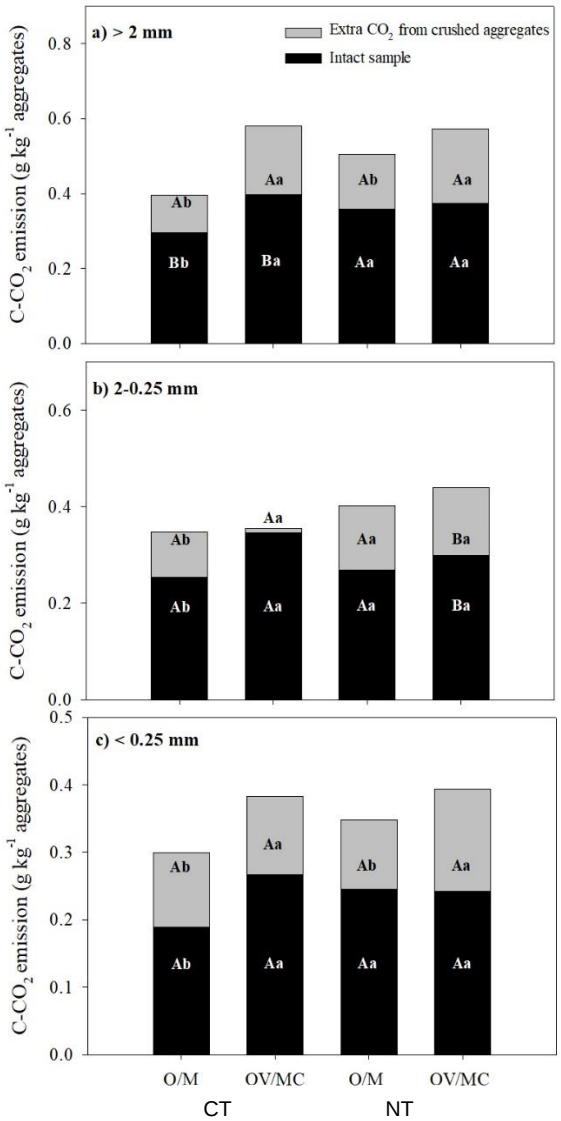


Figure 11. CO_2 emission from intact and crushed samples of large (a) and small (b) macroaggregates, and microaggregates (c) from 0–5 cm layer of sandy clay loam Acrisol subjected for 30 years to conventional tillage (CT) and no-tillage (NT) in combination to oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC). Uppercase letters compare tillage systems within the cropping system in each aggregate class. Lowercase letters compare cropping systems within the tillage system in each class of aggregate according to Tukey test ($p < 0.05$).

After crushing, 37 and 31% additional CO_2 was released on average for the three soil aggregate size classes from NT and OV/MC, compared to CT and O/M, respectively (Fig. 11 a,b,c). The additional CO_2 released from crushing was positively correlated ($p<0.001$) with the occluded POM-C (Fig. 12).

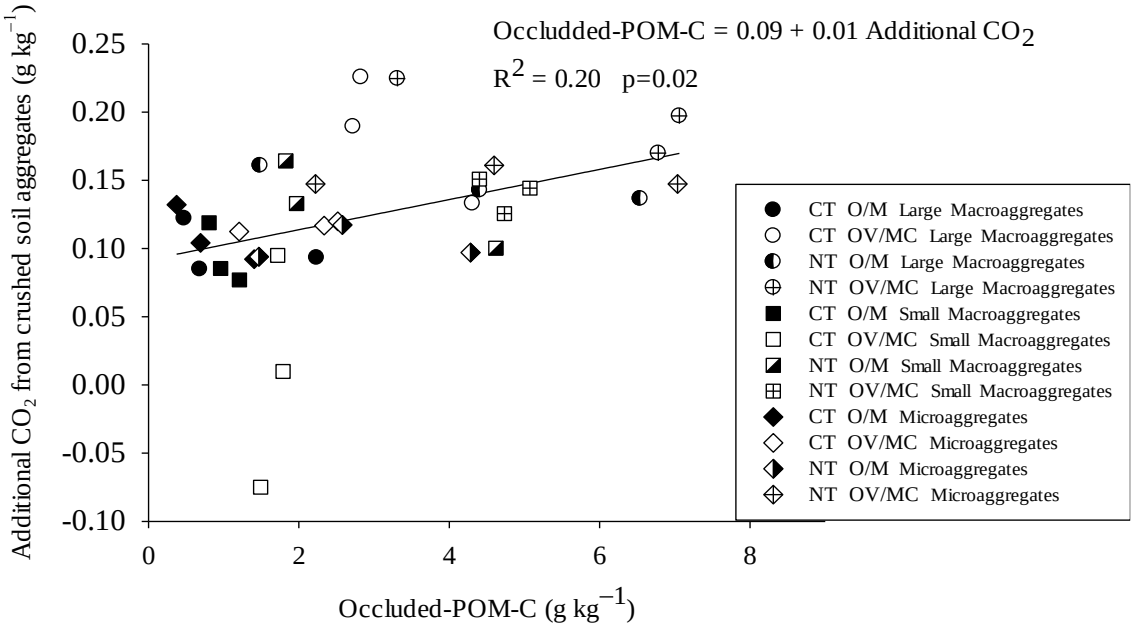


Figure 12. Regression between additional CO_2 released (crushed samples – intact samples) and carbon content in occluded particulate organic matter (occluded-POM-C) in large and small macroaggregates and microaggregates from 0–5 cm layer of sandy clay loam Acrisol subjected for 30 years to conventional tillage (CT) and no-tillage (NT) in combination to oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC).

4.4.3 Carbon pools, oxidation rates and mean residence time

The fast C pool of intact soil aggregates ranged from 1.0 to 2.2% with oxidation rates ranging between 7.99 to 15.65 $year^{-1}$. The slow pool represented the main C pool in soil aggregates, ranging from 97.8 to 99%, with oxidation rates between 0.001 and 0.05 $year^{-1}$ (Table 7).The oxidation rate of the fast and slow pools, on average for large and small soil macroaggregates, were 28 and 30% smaller under NT than CT. This lower oxidation rate of organic matter in macroaggregates under NT resulted in greater MRT of fast and slow pools by 0.03 and 40.7 years compared to CT.

Table 7. Pools, oxidation rates and mean residence time of carbon (MRT) of organic matter in large and small macroaggregates and microaggregates in 0–5 cm layer of a sandy clay loam Acrisol subjected for 30 years to conventional tillage (CT) and no-tillage (NT) in combination with two cropping systems: oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC).

	Class	CT		NT	
		O/M	OV/MC	O/M	OV/MC
---------- Intact soil samples ----------					
Pool fast (%)	Large macroaggregates	1.9±0.41	2.2±0.3	1.7±0.2	1.8±0.2
	Small macroaggregates	1.9±0.1	1.9±0.1	2.0±0.1	1.0±0.4
	Microaggregates	1.3±0.1	1.6±0.1	1.7±0.1	1.0±0.1
Pool slow (%)	Large macroaggregates	98.1±0.3	97.8±0.2	98.3±0.1	98.2±0.2
	Small macroaggregates	98.1±0.1	98.2±0.1	98.2±0.1	99.0±0.3
	Microaggregates	98.7±0.1	98.4±0.2	98.3±0.1	99.0±0.1
Rate fast ($year^{-1}$)	Large macroaggregates	11.40±0.2	12.26±2.7	9.34±1.3	7.99±1.3
	Small macroaggregates	12.66±1.4	15.65±2.1	10.09±2.3	10.70±1.2
	Microaggregates	9.15±2.0	11.29±0.6	13.87±0.3	10.46±0.4
	Soil	11.41±0.8	14.51±1.2	9.26±1.2	10.80±0.8
Rate slow ($year^{-1}$)	Large macroaggregates	0.03±0.00	0.02±0.00	0.01±0.00	0.01±0.00
	Small macroaggregates	0.02±0.00	0.03±0.00	0.03±0.00	0.02±0.00
	Microaggregates	0.001±0.00	0.004±0.00	0.004±0.00	0.003±0.00
	Soil	0.018±0.0002	0.032±0.002	0.011±0.003	0.012±0.002
MRT fast (years)	Large macroaggregates	0.09±0.00	0.09±0.02	0.11±0.01	0.13±0.02
	Small macroaggregates	0.08±0.01	0.07±0.01	0.09±0.02	0.09±0.01
	Microaggregates	0.11±0.00	0.09±0.01	0.11±0.00	0.08±0.00
	Soil	0.09±0.01	0.07±0.01	0.11±0.01	0.09±0.01
MRT slow (years)	Large macroaggregates	38.7±1.3	43.3±2.9	74.5±15.7	122.7±15.8
	Small macroaggregates	42.0±7.2	34.3±2.0	61.7±18.2	62.0±13.4
	Microaggregates	234.6±6.3	255.2±15.9	242.6±4.0	296.6±16.5
	Soil	109.15±5.11	87.53±19.20	126.85±10.56	131.64±15.37
---------- Crushed soil samples ----------					
MRT fast (years)	Large macroaggregates	0.08±0.01	0.07±0.01	0.09±0.02	0.09±0.01
	Small macroaggregates	0.08±0.01	0.11±0.03	0.09±0.00	0.08±0.02
	Microaggregates	0.10±0.00	0.09±0.01	0.10±0.01	0.10±0.00
	Solo	0.08±0.02	0.10±0.01	0.09±0.02	0.09±0.02
MRT slow (years)	Large macroaggregates	21.59±2.71	20.54±1.84	35.41±7.19	29.86±6.86
	Small macroaggregates	29.73±7.87	33.46±5.58	33.90±5.76	34.50±7.90
	Microaggregates	115.96±8.74	144.91±0.72	151.59±5.96	198.81±7.89
	Solo	57.78±1.37	69.32±2.44	73.72±4.50	89.97±7.13

[1] Standard deviation.

Under NT, the oxidation rate of fast and slow pools, on average for large and small soil macroaggregates were 5 and 17% smaller under OV/MC than O/M (Table 7). This lower oxidation rate of organic matter in macroaggregates resulted in greater MRT of fast and slow pools by 0.01 and 24.3 years, respectively, under OV/MC compared to O/M coupled with NT. However, cropping systems had remarkable effects on MRT of the slow pool of the microaggregates. Under NT, the MRT of microaggregates was 54 years greater under OV/MC than under O/M.

In both fast and slow pools of the three soil aggregate size classes was observed lower MRT in crushed than intact soil aggregates (Table 7). However, this negative effect of crushing aggregates on MRT was more evident for the slow pool (almost 50% on average of three aggregate classes) compared to fast pool (5% on average of three aggregate classes).

4.4.4 Contribution of C occlusion and mineral-organic association to mean residence time of the slow pool

The occluded POM-C was positively correlated with the MRT of the occluded C (Fig. 13). In large soil macroaggregates, carbon occlusion had a greater contribution to MRT under NT than CT (Fig. 15a), mainly when NT was coupled with legume cover crop-based cropping system (TRM = 100 years). The mineral-organic association explained more than 70% of the range of MRT in soil aggregates (Fig. 14). In microaggregates under NT, the contribution of mineral-organic association to MRT was greater under OV/MC (198.8 years) compared to O/M (151.6 years) (Fig. 15c).

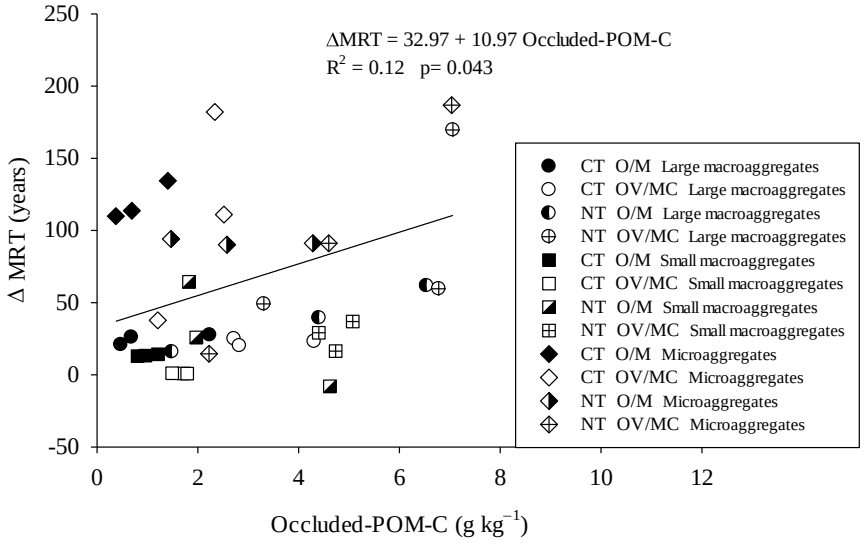


Figure 13. Regression between Δ mean residence time of carbon (crushed samples – intact samples) and carbon content in occluded particulate organic matter (occluded-POM-C) in (a) whole soil and (b) aggregates > 2, 2–0.25 and <0.25 mm from 0–5 cm layer of sandy clay loam Acrisol subjected for 30 years to conventional tillage (CT) and no-tillage (NT) in combination to oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC).

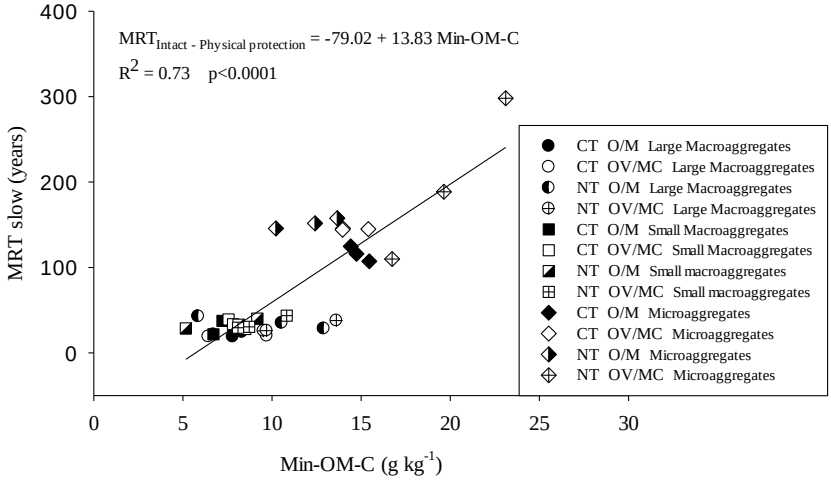


Figure 14. Regression between mean residence time of carbon (MRT) in slow pool of crushed samples and carbon content of mineral-associated organic matter (min-OM-C) in (a) whole soil and (b) large and small macroaggregates and microaggregates from 0–5 cm layer of sandy clay loam Acrisol subjected to conventional tillage (CT) and no-tillage (NT) in combination to oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC).

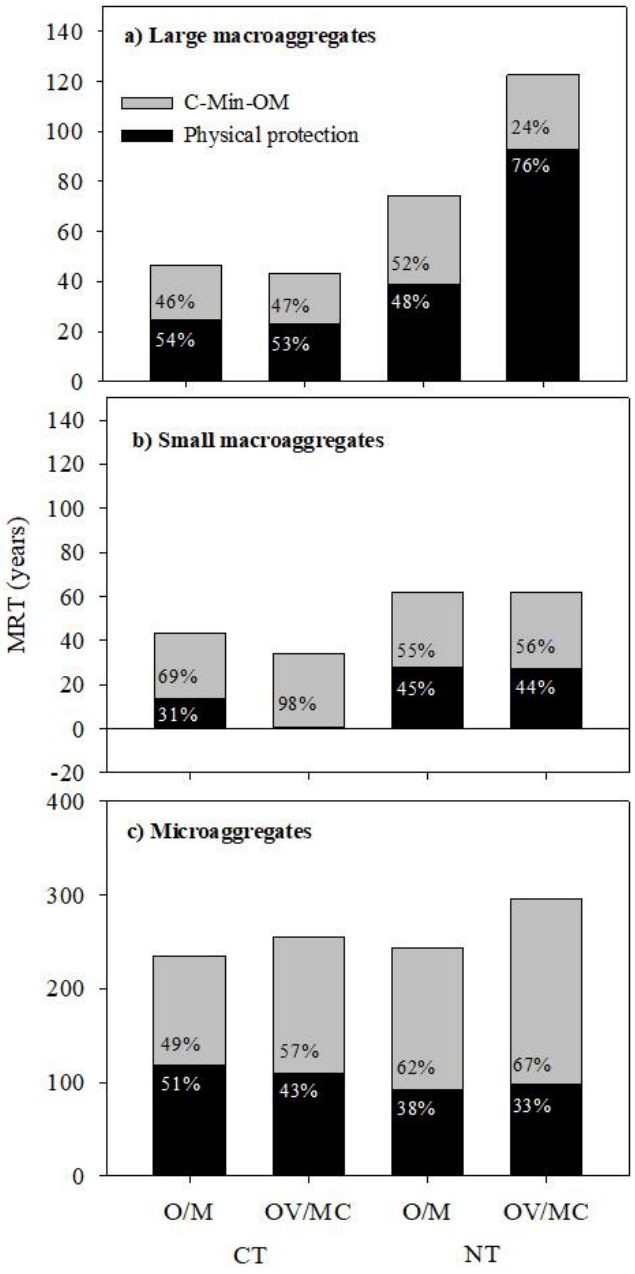


Figure 15. Contribution of physical protection (TRM $_{intact\ samples}$ – TRM $_{crushed\ samples}$) and mineral-organic association (TRM $_{crushed\ samples}$) to mean residence time (MRT) of carbon in large (a) and small (b) macroaggregates and microaggregates (c) from 0–5 cm layer of sandy clay loam Acrisol subjected to conventional tillage (CT) and no-tillage (NT) in combination to oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC).

4.5 Discussion

The SOC contains at least two pools with very different oxidation rates. The fast pool showed a MRT< 0.13 years suggesting a high oxidation rate and fast turnover rate and nutrient cycling. On the other hand, the MRT of the slow pool was much slower ranging from 23 to 297 years (Table 7).

The C oxidation rate was about 10 times higher in macroaggregates than microaggregates (Table 7) which translated into much longer MRT in micro than macroaggregates. Some studies suggested that the higher amount of particulate organic matter (Manna et al., 2013) and connectivity of pores in the macroaggregates compared to microaggregates (Rabbi et al., 2015) may favour a higher oxidation rate in the former. This agrees with other studies on isotopic techniques using ^{13}C natural abundance, that found turnover of 15-100 years for the C stored in macroaggregates and of 100-300 years for C of microaggregates (Angers & Giroux, 1996; Monreal et al., 1997; Puget et al., 2000; John et al., 2005).

The occluded POM-C was more abundant and the oxidation rate of the slow pool was slower in NT than CT (Tables 6 and 7) which results in slower turnover of C in macroaggregates from NT compared to CT as suggested by Six et al. (1999) and Du et al. (2015). In addition, C occlusion appears to be the main mechanism of C stabilization in large macroaggregates representing more than 70% of the MRT under NT and legume cover cropping (Fig. 13).

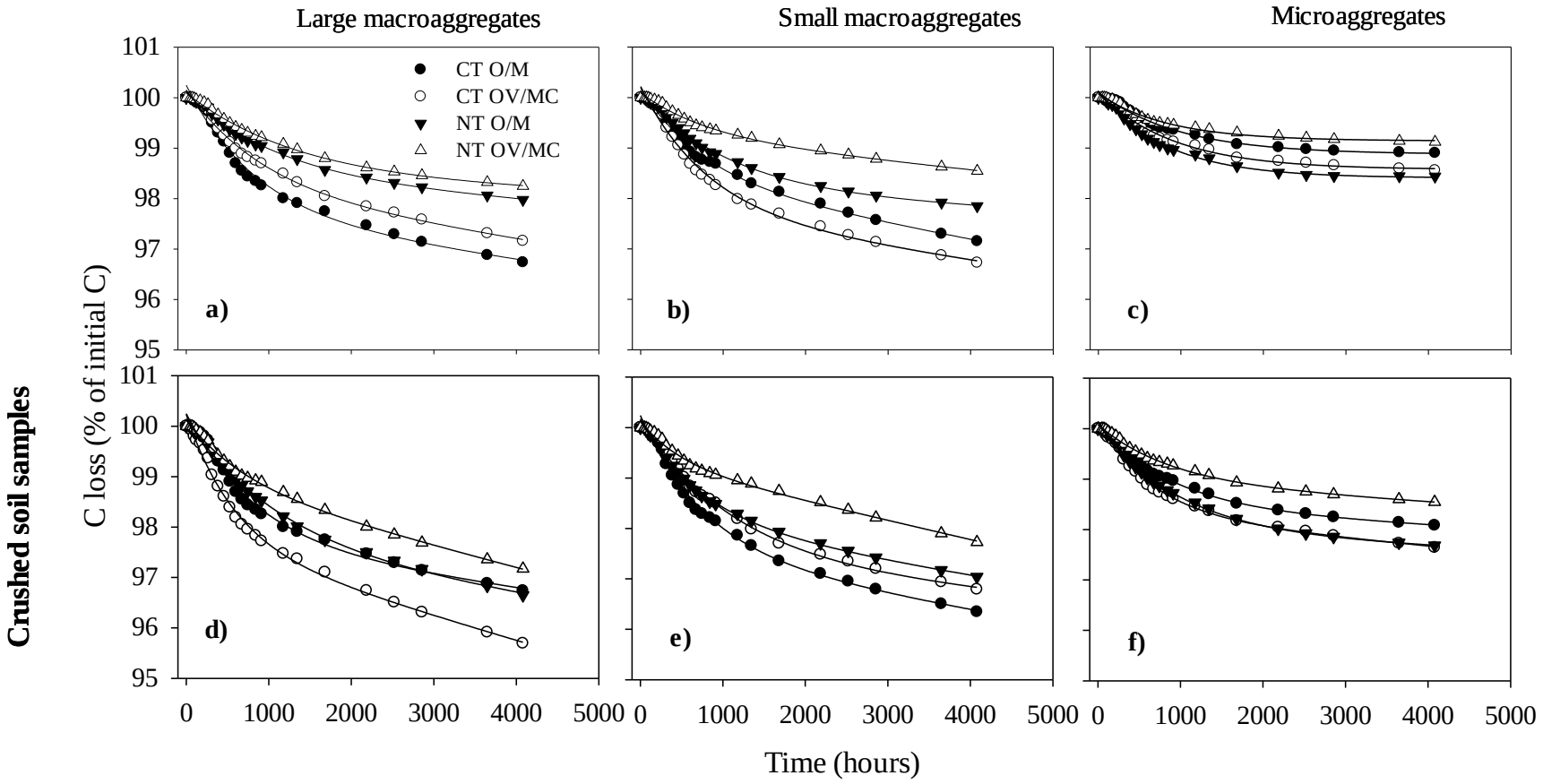
The legume cover crop-based cropping system had a strong positive effect on the mineral associated C in microaggregates, especially under NT. In this system combining legume cover cropping and NT, the mineral-organic association was the main mechanism of C stabilization in soil microaggregates, contributing to 67% of the MRT (Fig. 15c). The MRT of the slow pool of aggregates was largely explained by the content of mineral associated C which conceptually represents mineral-organic associations (Fig. 14), suggesting that this mechanism is the main driver of soil carbon stabilization in this subtropical Acrisol.

The slower turnover of the macroaggregates under NT is often invoked as a factor favouring the C enrichment in microaggregates (Six et al., 1999; Plaza-Bonilla et al., 2010). Our study suggests that this especially the case when

associated with legume cover crop-based cropping systems (Table 7). Our results also agree with the model of Cotrufo et al. (2015) which highlights the importance of labile compounds in the formation of stable mineral-associated SOM. In addition, recent studies observed that the sorption of N-rich microbial products to mineral surfaces suggests the possibility of increasing OM sequestration in stable mineral-organic particles by amending with N-rich substrates (Kopittke et al., 2017). Our results provided field-based evidence for the role of labile and N enriched residues through legume cover crops in stabilizing C through mineral-organic association. The formation of mineral-organic complexes reduces the oxidation rate of the slow pool and increases MRT, reaching 197 years in OV/MC under NT system (Fig. 14c).

4.6 Conclusions

Our results highlight the meaningful role of no-tillage and legume cover crops for long-term soil carbon stabilization in a subtropical Acrisol. The two management systems appear to induce different modes of actions. The C occlusion in large and small soil macroaggregates under NT slows down C turnover, mainly when combined with legume cover crop. On the other hand, the cropping systems with two legume cover crops results in longer C mean residence time compared to no-legume cropping systems under NT, mainly by favoring mineral-organic association in soil microaggregates. Our results provide field-based evidence for the role of labile and N enriched residues in stabilizing soil C through mineral-organic association, conferring long-term of soil carbon stabilization. The mineral-organic associations is the main driving mechanism of soil carbon stabilization in the subtropical Acrisol.



Supplementary 1. Cumulative loss of C as determined by its oxidation as a function of incubation time (from 30/01/15 to 21/07/15) for a repeat in intacts (a,b,c) and crushed (d,e,f) soil samples of large macroaggregatess (a,d), small macroaggregates (b,e) and microaggregates (c,f) from 0–5 cm layer of sandy clay loam Acrisol subjected to conventional tillage (CT) and no-tillage (NT) in combination to oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC).

5. HIGH CARBON SEQUESTRATION IN SUBTROPICAL SOIL PROFILES UNDER NO-TILLAGE WITH LEGUME COVER CROPS[1]

5.1 Abstract

The potential effects of no-tillage (NT) on soil organic carbon (SOC) sequestration may help Brazil meet its 37% greenhouse gas emissions reduction target by 2025. When combined with legume cover crops, NT could result in even greater SOC sequestration than NT alone. The objective of this study was to evaluate the SOC sequestration potential of NT and the contribution of legume cover crops and nitrogen (N) fertilization to this potential in both the superficial and sub-superficial soil layers of a subtropical Acrisol of Southern Brazil. Using a split-plot design, the long-term field experiment compared the effect of NT and conventional tillage (CT), with or without legume cover crops, and with or without mineral N fertilization. Thirty years of contrasting management systems resulted in large differences (up to 35 Mg ha^{-1}) in SOC stocks in the whole soil profile (0–100 cm) between treatments. The combination that provided the greatest increase in SOC was NT combined with two legume cover crops and N fertilization (1.15 Mg ha^{-1} $year^{-1}$ compared to CT, with no N fertilization or legume cover crop). Legume cover crops were twice as efficient in sequestering SOC as N fertilization alone, with 1 kg of residue input being converted to 0.30 kg of SOC. Overall, the variation in SOC stocks was explained largely by plant carbon input (R^2=80%) which varied with N fertilization and cropping system. About half of the SOC sequestration that occurred in this 30-year-old NT system was attributable to the increase in SOC stocks in the sub-superficial layer (30–100 cm), which was confirmed by the contribution of cover crops residues using carbon isotope signature (from 14.8 to ~17.5‰ in the 75–100 cm layer). Thus, the legume cover crop made a strong contribution to the potential of SOC sequestration in NT, and high rates of C accumulation occurred over a longer period in sub-superficial soil layers than previously believed.

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[1]Submitted to *Agriculture, Ecosystem & Environment.*

5.2 Introduction

The Paris climate agreement is aimed at holding global warming to below 2 °C by 2050 and to pursue efforts to limit it to 1.5 °C (Rogelj et al., 2016). Brazil made a voluntary commitment to reduce greenhouse gas (GHG) emissions by 37% by 2025 and established a climate plan, which encompasses a low carbon agriculture plan which includes NT farming as one of five thrusts for mitigation of GHG emissions (Brazil Ministry of the Environment, 2015).

No-tillage is the basis of conservation agriculture and is essential to the sustainability of agriculture activities in tropical and subtropical regions (Paustian et al., 1997; Bayer et al., 2000; Lal et al., 2007). A range of studies from tropical and subtropical environmental (Bayer et al., 2006a) show that NT can result in mean atmospheric C sequestration rates of 0.35 and 0.48 Mg ha^{-1} $year^{-1}$, respectively, in comparison to conventional tillage.

The effect of NT on SOC accumulation is also dependent on the amount and diversity of the crops grown (Diekow et al., 2005b; Martins et al., 2012b; Raphael et al., 2016). Legume cover crops play an essential role in SOC accumulation under NT, either through the biomass inputs (shoot and root) associated with these plant species or through the symbiotically fixed N which becomes available and increases the grain and biomass production of cash crops grown in succession (Amado et al., 2006). The higher quality (N content and soluble fractions) of the biomass of legume cover crops, whether cultivated alone or intercropped with other species, may improve the efficiency of the microorganisms to accumulate C in the soil (Cotrufo, 2013). However, there is no consensus on the effect of nitrogen fertilization on soil SOC stocks because, in spite of the positive effect it has on plant biomass addition (Mack et al., 2004; Kirchmann et al., 2013), it can cause decomposition of the existing C (Khan et al., 2007).

Soil depth should be considered carefully in evaluating SOC sequestration under NT. In temperate soils of North America and Europe, the gain of SOC in superficial layer under NT may be compensated by gains in sub-superficial layers under CT (Angers et al., 1997; Baker et al., 2007; Blanco-Canqui et al., 2011;

Dimassi et al., 2014). By contrast, some studies carried out in tropical and subtropical soils in Brazil have shown that sampling of the superficial soil can lead to underestimation of the potential of SOC accumulation in NT (Boddey et al., 2010; Alburquerque et al., 2015; Miranda et al., 2016). In those studies sampling of the 0–100 cm layer resulted in SOC sequestration rates that were 59% (Boddey et al., 2010) and 100% (Albuquerque et al., 2015) higher than for soil sampling done at a depth of 0–30 cm. The accumulation of C in sub-superficial layers under NT is important in Brazilian tropical and subtropical soils, especially in the case of cropping and rotation systems that incorporate cover crops (Boddey et al., 2010). A higher volume of rainfall may favor the percolation of organic compounds and contribute to the potential for SOC accumulation in sub-superficial layers of tropical soils under conservation management systems (Hobley et al., 2016). In addition, free-draining soils (Miranda et al., 2016) with Bt horizons (Torres-Sallan et al., 2017) and functional groups on the surface of iron and aluminum oxides that interact strongly and stabilize organic matter (Lawrence et al., 2015) point to considerable potential for SOC accumulation in sub-superficial layers.

We hypothesized that over the long term, legume cover crops would make a strong contribution to the potential of SOC sequestration in NT, and that C accumulation could occur at higher rates in sub-superficial layers of subtropical soils in long-term. Our objective was to evaluate the potential that NT offers for SOC accumulation as well as the contribution of legume cover crops and nitrogen (N) fertilization to this potential in both superficial and sub-superficial layers of a subtropical Acrisol.

5.3 Materials and methods

5.3.1 Description of experiment

The study was conducted in a long-term experiment (30 years) at the Agronomic Experimental Station of the Federal University of Rio Grande do Sul in the municipality of Eldorado do Sul–RS (30º06'S, 51º40'W, elevation 96 m). The climate is subtropical (Cfa according to the Köppen classification), with a mean temperature of 19.4 °C and annual rainfall of 1440 mm. The soil was classified as

sandy clay loam granite-derived Acrisol (FAO, 2015), with a loamy clay texture in the superficial layer. However, the clay content in the soil profile increases, from 217 g kg^{-1} in the 0–5 cm layer to 394 g kg^{-1} in the 20–30 cm layer, reaching 511 g kg^{-1} in the 75–100 cm layer. The main minerals in the clay fraction are kaolinite (720 g kg^{-1}) and iron oxides (109 g kg^{-1}) (Bayer et al., 2001).

Until 1969, the study area was under native pasture (mainly *Paspalum* spp. and *Andropogon* spp.). In the following 16 years, the soil was tilled by plowing and disking for annual crops, leading to serious problems of soil degradation, until the experiment was initiated in 1985.

The experiment included two soil tillage systems, arranged in main plots of 15 × 20 m: CT and NT. Each tillage system was composed of three cropping systems in subplots of 5 × 20 m: black oats (*Avena strigose* Schreb) / maize (*Zea mays* L.) (O/M), vetch (*Vicia sativa* L.) / maize (V/M) and oats + vetch / maize + cowpea (*Vigna unguiculata* (L.) Wald) (OV/MC). These tillage systems combined with cropping systems were managed with two levels of fertilization, 0 and 180 kg ha^{-1} of N-urea (0N and 180N), applied in strips in the maize crop, characterizing the sub-subplots (5 × 10 m). The experimental design consisted of randomized blocks with split-split plots and three replications.

Winter crops were established in April–May of each year. Oats, when grown alone, were seeded at a rate of 80 kg ha^{-1}. When oats were grown with vetch, oats were seeded at 30 kg ha^{-1}, and vetch at 50 kg ha^{-1}. For vetch cultivated alone, 80 kg ha^{-1} was used. In the OV/MC system, cowpea was sown 15 to 20 days after the maize, between the lines of this crop, in pits located 40 cm apart. After the maize was harvested, the cowpea could develop fully, remaining until mechanical management to permit the establishment of oats and vetch.

The CT plots were ploughed to a furrow-depth of 17 cm once a year in spring before maize sowing using a three-disk plough and harrowed twice to a depth of 10 cm using a disk harrow mixing the crops residues in this layer. At the same time, glyphosate-based herbicide (Roundup, Monsanto) was applied in the NT plots at 1.4 kg ha^{-1} with respect to final glyphosate concentration, and two or three days later the winter cover crops were managed with a knife-roller and the

aboveground residues left on the soil surface. In NT, soil disturbance occurred only in the sowing line, and the residues of the cover crop were left on the soil surface.

Maize was planted with NT sowing in September–October, with between-row spacing of 90 cm and a sowing rate designed to obtain 50–70 thousand plants per hectare. The fertilizer rate applied in maize was 21.5 and 41.5 kg ha^{-1} of P and K (50 and 50 kg ha^{-1} of P_2O_5 and K_2O), respectively.

Annual C addition by cropping systems (aboveground and root, with roots considered to be 30% of the aboveground parts) was calculated using the data compiled by Zanatta et al. (2007) based on a survey from the beginning of the experiment until 2006 and updated to 2014 (Fig. 16).

5.3.2 Soil bulk density and SOC concentrations and stocks

Soil samples from eight depths (0–5, 5–10, 10–15, 15–20, 20–30, 30–50, 50–75 and 75–100 cm) were collected in September 2014 prior to soil tillage for maize crop establishment. Trenches were excavated with a backhoe to permit measurement of soil bulk density in duplicate by the volumetric ring method (Blake & Hartge, 1986) only in sub-subplots with no nitrogen fertilization, assuming that nitrogen fertilization did not modify soil density.

Soil sampling for analysis of SOC was carried out in the 0 and 180 N sub-subplots, in the same soil layers as for the evaluation of bulk density. After soil samples were air-dried, ground and passed through a 2-mm mesh sieve, a subsample of approximately 2 g was further crushed in an agate mortar to pass through a 250-µm mesh. Determination of SOC concentrations via dry combustion was performed in an elemental analyzer (FlashEA 1112, Thermo, Electron Corporation, Milan, Italy).

SOC stocks down to depths of 30 and 100 cm were calculated using the equivalent soil mass approach (Ellert & Bettany, 1995), which considers equal masses of soil between treatments. The soil under CT OM 0N was used as the reference for the equivalent mass. The annual SOC sequestration rate was calculated as the difference between the SOC stocks of the treatments and the

reference, divided by the time elapsed since the implementation of the experiment, i.e. 30 years.

To assess the contribution of the cover crops to the SOC in the soil profile, isotopic abundance (^{13}C) was determined for samples from the contrasting treatments in the sub-subplots with no nitrogen fertilization: CT OM, CT OV/MC, NT OM and NT OV/MC. The isotopic abundance values for different soil layers were obtained by evaluating the soil samples from the 0–5, 20–30 and 75–100 cm depths in an elemental analyzer (FlashEA 2000, Thermo Fisher Scientific, Bremem, Germany) coupled to an isotope ratio mass espectometer (IRMS) (Delta Advantage, Thermo Fisher Scientific, Bremen, Germany).

5.3.2 Historical evaluation of SOC stocks and sequestration

We compared our results obtained in 2014 with those obtained in five previous studies conducted since the beginning of the experiment to build a historical assessment of SOC, specifically for the 0-20 cm layer, and for the without N treatment, which was the depth and N level common to all studies. Previous SOC measurements were carried out in in 1985 (Medeiros, 1988), in 1990 (Bayer & Mielniczuk, 1997), in 1994 (Bayer et al., 2000), in 1998 (Lovato et al., 2004), and in 2003 (Zanatta et al., 2007). All the experimental methodologies were similar to those used in 2014, with the exception of the analytical C method. Therefore, SOC data from previous studies using the Walkley-Black analytical method (Nelson & Sommers, 1996) were corrected by a factor of 0.9422 (Zanatta et al., 2007) . The SOC dataset for previous years was also recalculated from the original values using the equivalent soil mass method.

5.3.3 Statistical analyses

After normality was analyzed by means of the Kolmogorov-Smirnov test and homogeneity of variance by the Levene test, the data were subjected to analysis of variance (ANOVA) and, significant results ($p < 0.05$) were compared using the Tukey test ($p < 0.05$). The MIXED procedure was used to compare the effects of tillage methods (T), crop systems (CC), nitrogen fertilization (N) and soil layers (L)

on the response variables. This procedure considers the main factors and their interactions as fixed factors and the block variable and the experimental errors as random variables. All analyses were performed using the SAS statistical package® v.9.4 (Statistical Analysis System Institute, Cary, North Carolina).

The statistical model used in the analysis of variance to evaluate C concentration was as follows:

$Y_{ijklm} = \mu + B_i + T_j + Error_{(ij)} + C_k + T_jC_k + Error_{(ijk)} + N_l + T_jN_l + C_kN_l + T_jC_kN_l + Error_{(ijkl)} + L_m + Error_{(im)} + T_jL_m + C_kL_m + N_lL_m + T_jC_kL_m + T_jN_lL_m + C_kN_lL_m + T_jC_kN_lL_m + Error_{(ijklm)}$.

Where μ = general mean of the experiment; B = block (i = 1, 2, 3); T = tillage systems (j = 1, 2); C = cropping system (k = 1, 2, 3); N = nitrogen fertilization (l = 1, 2); L = soil layers (m = 1, 2, 3, 4, 5, 6, 7, 8), and Error = experimental error. To assess the SOC stocks and sequestration rates, the variable soil layers and its associated errors were removed from the statistical model.

The least difference significant (LSD) was calculated multiplying the mean standard error by the value obtained in Tukey table ($p<0.05$). For SOC concentration was considered the greater values of LSD in order to be more rigorous in comparing averages and facilitate the presentation of results.

5.4 Results

5.4.1 Historical carbon input data

The inclusion of legume cover crops and nitrogen fertilization caused an approximately twofold increase in the annual C input, which amounted to 4.98 and 10.0 Mg ha^{-1} $year^{-1}$ in the O/M 0 N and OV/MC 180 N systems (Fig. 16), respectively. The effect of tillage systems was not significant (Fig. 16).

In systems where nitrogen fertilizer was applied to the maize crop, the impact of legume cover crops on C addition was smaller because C inputs from maize were similar for the three cropping systems. In all cropping systems, maize made a significant contribution in terms of C addition, although the amount of C added was greater in systems with nitrogen fertilization (53−67% of annual

addition) than in those without nitrogen fertilization (42−59% of annual addition) (Fig. 16).

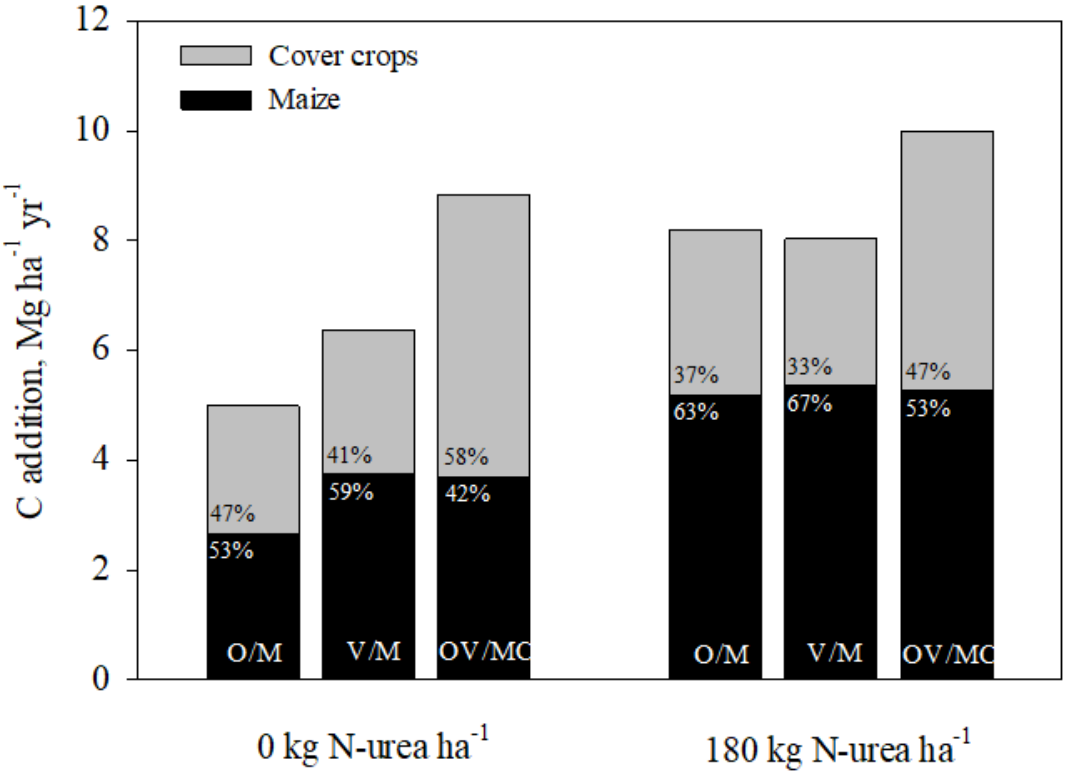

Figure 16. Mean annual C addition distribution across oat/maize (O/M), vetch/maize (V/M) and oat + vetch/maize + cowpea (OV/MC) cropping systems subjected to two N-urea rates (0 N = 0 kg ha^{-1} and 180 N = 180 kg ha^{-1}). Average values of two tillage systems (no-tillage and conventional tillage).

5.4.2 C accumulation in the superficial soil layer over time

Thirty years of contrasting management systems resulted in a large difference in SOC stocks in the 0–20 cm layer (Fig. 17a)**.** From 1985 to 2014, in systems with no nitrogen fertilization, the conventional management system (CT O/M) resulted in a decrease of 3.8 Mg ha^{-1} in SOC stocks of the 0−20 cm layer, with a greater reduction in the first 5 years (Fig. 17a). In contrast, all systems with legume cover crops showed a positive SOC balance over the 30 years. In NT, the OV/MC system increased SOC stocks by 5.6 Mg ha^{-1} to 41.8 Mg ha^{-1} (Fig. 18a).

The SOC sequestration rate for the 20-cm soil layer reached its maximum level between five and nine years after the experiment began, reaching 1 Mg ha^{-1} $year^{-1}$ in the NT OV/MC system (Fig. 17b). After this peak in SOC sequestration, the rates decreased exponentially over time. However, it is noteworthy that even after 30 years, all systems continued to accumulate SOC in the 0−20 cm soil layer, at rates ranging from 0.05 to 0.32 Mg ha^{-1} $year^{-1}$ according to the tillage and cropping systems.

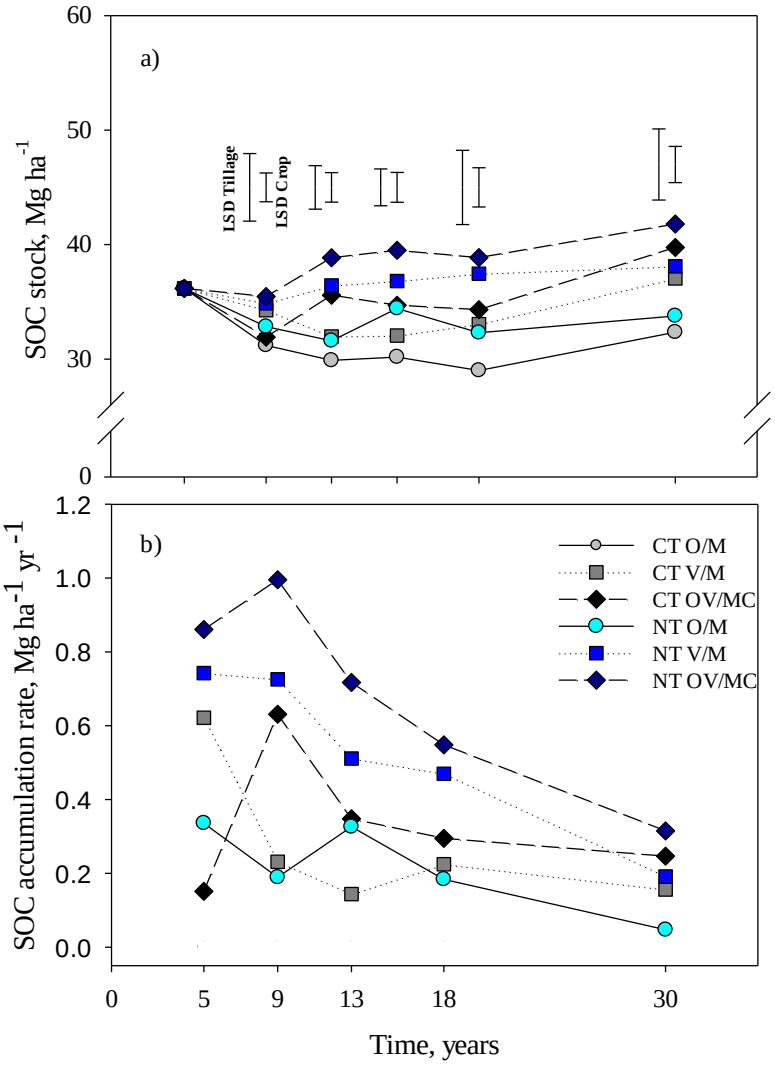

Figure 17. Temporal evolution of (a) SOC stocks, (b) SOC accumulation rates in the 0–20 cm soil layer as affected by oat/maize (O/M), vetch/maize (V/M) and oat + vetch/maize + cowpea (OV/MC) cropping systems with no addition of inorganic N (0 N), under CT and NT systems. The SOC accumulation rate was calculated in relation to the reference CT O/M 0 N treatment (baseline). The vertical bars indicate the least significant difference (LSD) ($p < 0.05$).

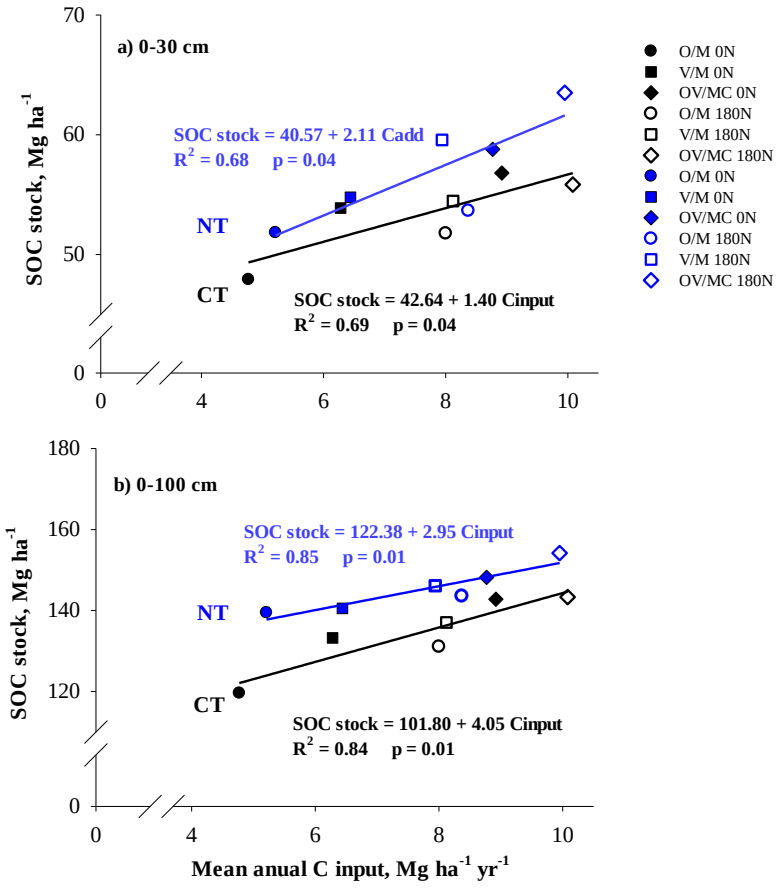

Figure 18. Relationship between soil organic C (SOC) stocks in the 0–30 and 0–100 cm soil layers after 30 years, and C addition by cropping systems [oat/maize (O/M), vetch/maize (V/M) and oat+vetch/maize+cowpea (OV/MC)] subjected to two N-urea rates, 0 kg ha^{-1} (0 N) and 180 kg ha^{-1} (180 N), under conventional tillage (CT) and no-tillage (NT) systems.

5.4.3 Whole profile (0–100 cm) soil C stocks after 30 years

5.4.3.1 Tillage systems

The increase in soil C content in NT occurred mainly in the superficial soil layer (0−5 cm) and is also reflected in the higher SOC stocks observed in the 0−30 cm layer when compared to CT (Table 8). SOC stocks between tillage systems ranged from 47.92 to 63.33 Mg ha^{-1} in the 0−30 cm layer, and from 119.59 to 154.20 Mg ha^{-1} in the 0−100 cm layer (Table 8). The largest differences in the

0−100 cm layer between CT and NT were 13 and 11 Mg ha^{-1} in the O/M 180N and OV/MC 180N systems, respectively, which led to significant increments of 0.42 and 0.36 Mg ha^{-1} $year^{-1}$ in SOC sequestration rates (Table 9).

Table 8. SOC concentration[1,2] in eight soil layers and SOC stocks[3] in two soil layers of a sandy clay loam Acrisol subjected to conventional tillage (CT) and no-till (NT) for three cropping systems: oat/maize (O/M), vetch/maize (V/M) and oat+vetch/maize+cowpea (OV/MC); with two N-urea rates (0 N = 0 kg ha^{-1} and 180 N = 180 kg ha^{-1}).

Fertilization	Tillage	Crop	SOC concentration								SOC stocks	
			Soil layer (cm)									
			0–5	5–10	10–15	15–20	20–30	30–50	50–75	75–100	0–30	0–100
			g kg^{-1}								Mg ha^{-1}	
0 N	CT	O/M	10.6	9.5	9.4	9.8	9.1	8.7	6.4	5.4	47.9	119.6
		V/M	12.2	12.0	10.8	10.0	9.9	8.8	7.3	6.2	53.8	133.2
		OV/MC	13.4	11.7	12.6	10.6	10.0	9.2	8.3	6.6	56.7	142.9
	NT	O/M	15.0	8.9	8.8	8.8	9.4	9.7	8.4	6.5	51.7	139.5
		V/M	19.4	10.5	8.8	8.4	9.8	9.6	8.1	6.4	54.6	140.5
		OV/MC	20.9	11.7	9.8	9.4	10.6	9.9	8.5	6.7	58.6	148.2
180 N	CT	O/M	11.3	10.0	10.6	9.4	10.4	8.9	7.3	6.2	51.7	131.1
		V/M	14.1	12.1	10.4	9.2	9.9	9.4	7.8	6.1	54.4	137.1
		OV/MC	12.9	11.2	11.8	10.3	10.4	9.9	8.2	6.5	55.8	143.3
	NT	O/M	17.2	9.8	9.2	8.7	10.1	10.4	8.5	6.5	53.5	143.6
		V/M	22.9	12.0	9.1	8.2	10.3	9.8	8.1	6.4	59.4	146.1
		OV/MC	25.9	13.3	9.8	8.8	10.0	10.3	8.6	6.7	63.3	154.2
LSD		Tillage				[1,2]	1.4			[3]	5.6	12.9
(p < 5%)		Crop					1.7				5.5	15.7
		N fertilization					1.1				3.8	7.2
		Layer					2.0				-	-

The least significant difference (LSD) (p < 0.05) was made considering two triple interaction for SOC concentration (see below numbers 1 and 2) and one triple interaction for the both SOC stocks 0–30 cm and 0–100 cm (see below number 3):(1) Interaction between tillage and cropping systems and soil layers. Comparisons of SOC concentrations were performed on the average of two nitrogen fertilization rates;
(2) Interaction between tillage systems, nitrogen fertilization and soil layers. Comparisons of SOC concentrations were performed on the average of the three cropping systems;
(3) Interaction between tillage and cropping systems and nitrogen fertilization for comparisons of SOC stocks.

5.4.3.2 Cropping systems

The use of two legume cover crops (one in winter and another in summer - OV/MC) in NT increased SOC concentrations in the 0−10 cm layer by 39% compared to exclusive grass cultivation (O/M), and this increase explained the difference in SOC stocks in the 0−30 cm layer (Table 8). In the 0−100 cm layer, OV/MC increased the SOC stocks by an average of 14 Mg C ha^{-1} relative to O/M (Table 8). For both the 0−30 and 0−100 cm soil layers, annual C inputs explained between 68% and 85% of the SOC stock range in NT and CT (Fig. 18a and b). Considering the relation between the 0−30 and 0−100 cm soil layers, we can see that the SOC sequestration rate was 62% greater in the 0−100 cm layer compared to the 0−30 cm layer (Fig. 19). In the 0−100 cm layer, the SOC sequestration rate was 0.38 Mg ha^{-1} $year^{-1}$ greater in OV/MC than in O/M (Table 9) on average, for CT 180N and NT 180N. Thirty years of NT and OV/MC with 180 kg ha^{-1} of N-urea resulted in a SOC sequestration rate that was 1.15 Mg ha^{-1} $year^{-1}$ higher in the 0−100 cm depth compared to CT and O/M without nitrogen fertilization (Fig. 20). A marked increase was observed in the SOC concentration in superficial layers and increases were observed throughout the soil profile down to a depth of 100 cm.

Table 9. SOC accumulation rates in a sandy clay loam Acrisol subjected to conventional tillage (CT) and no-till (NT) for three cropping systems: oat/maize (O/M), vetch/maize (V/M) and oat+vetch/maize+cowpea (OV/MC); with two N-urea rates (0 N = 0 kg ha^{-1} and 180 N = 180 kg ha^{-1}).

Fertilization	Tillage	Crop	SOC accumulation rate	
			0–30	0–100
			------Mg ha^{-1} yr^{-1}-------	
0 N	CT	O/M	0.00	0.00
		V/M	0.15	0.42
		OV/MC	0.25	0.80
	NT	O/M	0.13	0.66
		V/M	0.22	0.70
		OV/MC	0.26	0.95
180 N	CT	O/M	0.13	0.38
		V/M	0.22	0.58
		OV/MC	0.36	0.79
	NT	O/M	0.19	0.80
		V/M	0.38	0.88
		OV/MC	0.51	1.15
LSD*		Tillage	0.18	0.36
(p< 5%)		Crop	0.20	0.41
		N fertilization	0.15	0.20

* Least significant difference (p < 0.05) of interaction between tillage, cropping systems and nitrogen fertilization for comparisons of SOC accumulation rate.

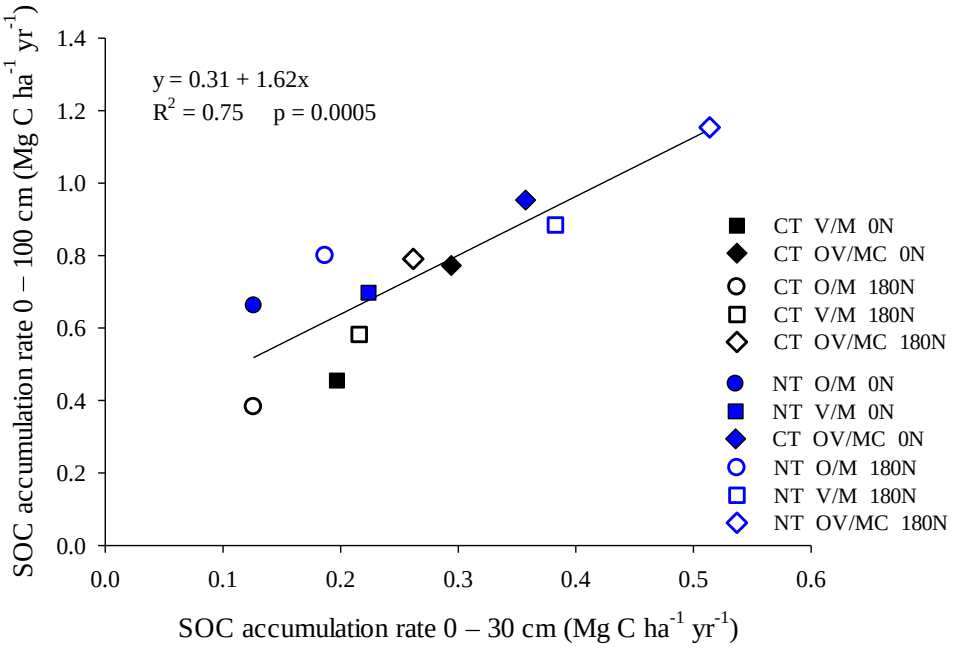

Figure 19. Regression of soil C accumulation between 0–30 cm and 0–100 cm depth under conventional tillage (CT) and no-tillage (NT) for three cropping systems [oat/maize (O/M), vetch/maize (V/M) and oat+vetch/maize+cowpea (OV/MC)]; with two N-urea rates (0 N = 0 kg ha^{-1} e 180 N = 180 kg ha^{-1}).

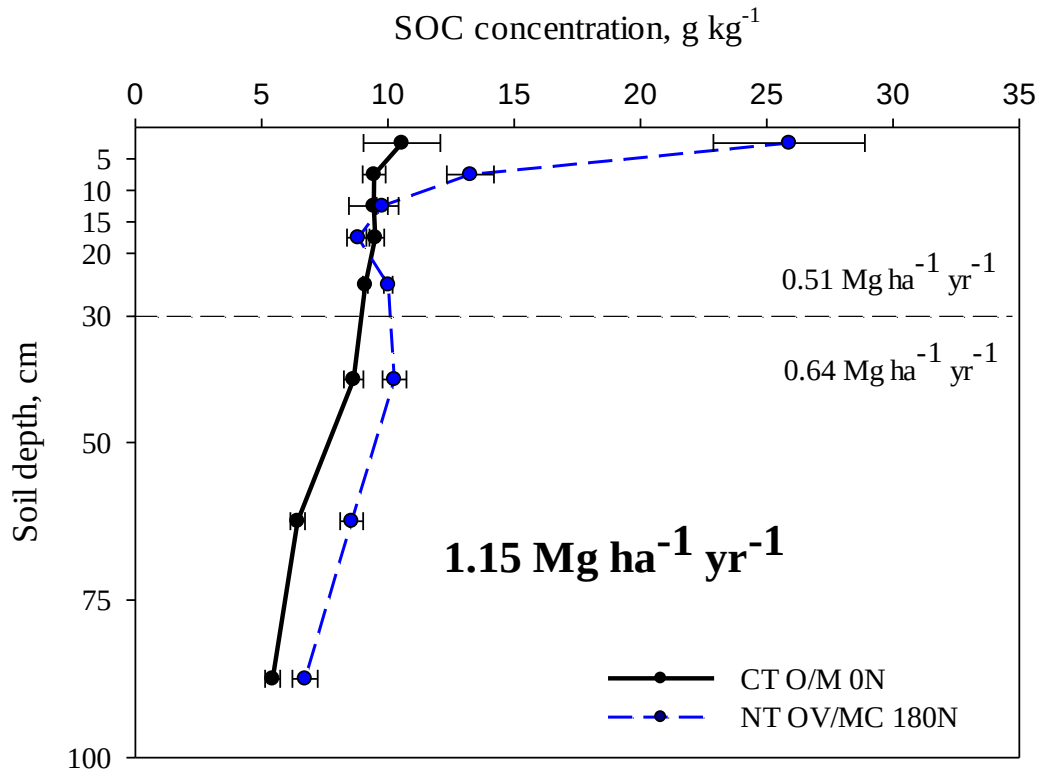

Figure 20. Concentration of SOC at 0–100 cm depth in a sandy clay loam Acrisol under NT OV/MC 180N compared to CT O/M 0N.

The $\delta^{13}C/^{12}C$ of the soil under natural vegetation was close to −15.9‰ owing to the predominance of C4 plants. A reduction in the carbon isotope signature was observed due to the contribution of C3 plants (oat, vetch and cowpea) to SOC during the 30 years of experiment (Table 10). In the plots with OV/MC, the reduction of $\delta^{13}C/^{12}C$ due to C3 plants was significantly higher in the 0−5 (mean of 19.9‰) and 75−100 cm (mean of 17.6‰) soil layers (Table 10). The O/M system increased the signature to −15 ‰, which points to a greater contribution of maize to SOC accumulation.

Table 10. Natural abundance of ^{13}C in the 0–5, 20–30, and 75–100 cm layers of a sandy clay loam Acrisol subjected to conventional tillage (CT) and no-tillage (NT) with oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC) and no N fertilization.

Depth (cm)	δ $^{13}C/^{12}C$ (‰)			
	Tillage	Crop		NG
0–5				-15.9±0.2[1]
	CT	O/M	–15.0± 1.0[1] Aa[2]	
		OV/MC	–18.9± 0.6 Ab	
	NT	O/M	–15.1± 0.7 Aa	
		OV/MC	–20.8± 0.4 Bb	
20–30				-14.4±0.4
	CT	O/M	–15.4± 0.2 Aa	
		OV/MC	–15.8± 0.4 Aa	
	NT	O/M	–15.5± 0.8 Aa	
		OV/MC	–16.5± 0.9 Aa	
75–100				-14.8±0.4
	CT	O/M	–14.9± 0.2 Aa	
		OV/MC	–18.4±0.6 Bb	
	NT	O/M	–15.0± 0.3 Aa	
		OV/MC	–16.7± 0.8 Ab	

[1] Standard deviation; [2] uppercase letters compare tillage systems and lowercase letters compare cropping systems; means with the same letter do not differ significantly according to Tukey test ($p < 0.05$).

5.4.3.3 Nitrogen fertilization

Nitrogen fertilization with urea at a rate of 180 kg ha^{-1} led to an average increase of 16% in SOC concentration in the first 10 cm of soil. This significant difference in SOC at the superficial resulted in a significant effect in SOC visible down to 30 and 100 cm. This increase was on average 2.5 Mg ha^{-1} at 30 cm and 5.3 Mg ha^{-1} at 100 cm (Table 8). In CT O/M, SOC stocks increased from 119.6 Mg C ha^{-1} to 131.1 Mg C ha^{-1} (Table 8) due to addition of 180 kg ha^{-1} of N-urea, leading to SOC accumulation of 0.38 Mg ha^{-1} $year^{-1}$ (Table 9).

However, the SOC accumulation per unit of C added by N-urea was lower than the SOC accumulation associated with legume cover crops (Fig. 21). On average, SOC accumulation attributable to the use of legume cover crops was twice as high as that for nitrogen fertilization. In other words, each kg of C input by the legume cover crops resulted in the accumulation of 0.38 kg of SOC (0.22–0.44 kg SOC), compared with only 0.20 kg SOC (0.09–0.31 kg SOC) for nitrogen fertilization.

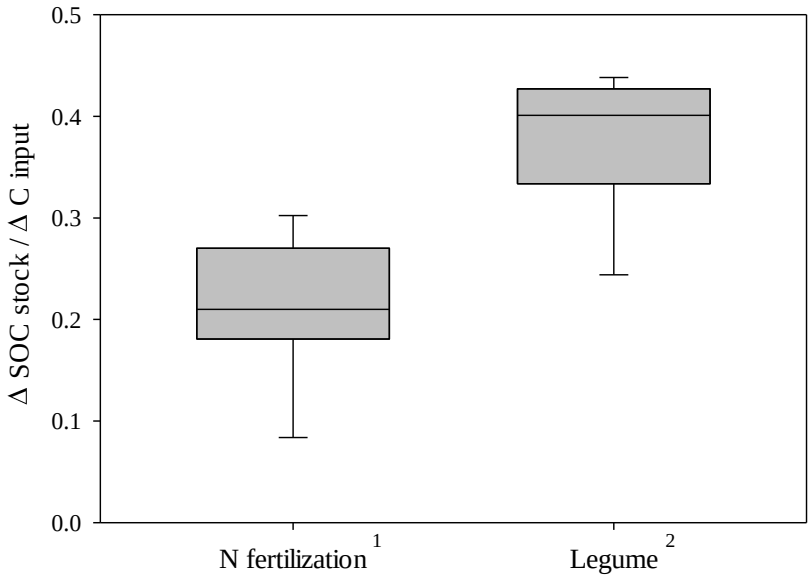

Figure 21. Relation between SOC stocks and C inputs derived from N fertilization or legume crops. CT O/M 0N served as a reference for the calculation of Δ SOC stock and Δ C input. N fertilization= SOC stocks with N addition – SOC stocks with no N addition / difference between C input from O/M 180 N and 0 N; Legume=SOC stocks V/M or OV/MC with no N addition – O/M with no N addition / difference between C input from V/M and OV/MC and O/M;

5.5. Discussion

Thirty years of contrasting management systems resulted in large differences (up to 35 Mg ha^{-1}) in SOC stocks in the whole soil profile (0–100 cm) (Table 8). Small accumulations of SOC were also observed in sub-superficial layers to a depth of 100 cm, and, when taken into consideration, this increased the stocks and the sequestration rate of SOC in NT compared to CT (Table 8). This is in agreement with recent studies (Varvel & Wilhelm, 2010; Kibet et al., 2016) which suggest that NT affects not only the superficial layer, but also the distribution of SOC throughout the soil profile, contributing to SOC sequestration at depth.

Cropping systems increased the potential for SOC sequestration when legume cover crops were included (O/M < V/M < OV/MC), with the highest potential recorded when two legumes (OV/MC) were cultivated. The ability of legume cover crops to capture and supply N for subsequent crops or intercrops increases N utilization and crop yield (Jensen, 1996). In addition to favoring the supply of N and increasing C inputs from maize or a subsequent crop, legume

cover crops add C from their own residues (Lovato et al., 2004; Amado et al., 2006), which includes inputs from roots and affects the SOC in deeper soil layers. Also, the greater lability of the legume cover crop residues may contribute more microbial residue, which is stabilized by chemical bonding with the mineral soil matrix (Cotrufo et al., 2013; Cotrufo et al., 2015). Around 80% of the difference in SOC stocks is explained by carbon inputs from cropping systems and N fertilization in each tillage system (Fig. 18a and 18b).

Nitrogen fertilization had a positive effect on SOC stocks due to the larger amount of residue (shoot + root) added by the crop plants. However, the accumulation of SOC per unit of C added due to N fertilization is about half of that of legume (Fig. 21). This is because nitrogen fertilization releases a large amount of N all at once, which can often cause decomposition of the existing C. This is known as a priming effect (Khan et al., 2007).

Considering only the superficial soil layer (0–20 cm Fig. 17), the pattern of high initial rates of SOC sequestration (first 5–9 years) and the subsequent decline suggests that the adoption of conservation management systems is a limited, short-term strategy for mitigating the increase in atmospheric C concentrations. However, more than half of the SOC sequestration after 30 years was attributable to the increase in C stocks in the sub-superficial layer (30–100 cm) (Fig. 19), which was linked to cover crop residues (shoot and root) through carbon isotope signature. The variation of isotopic signature, between −15 and −20‰, suggests that C from the cropping systems was added to C from the native field, which was predominantly composed of C4 plants (close to −14‰), and this which changed its signature (Table 10). The more negative values in the OV/MC system in relation to O/M in the superficial and sub-superficial layers are due to the greater contribution of residues of C3 plants (oats, vetch and cowpea) which favored the SOC accumulation up to a depth of 100 cm.

Our results related to SOC accumulation in different soil depths agree with the findings of other studies conducted in tropical (Miranda et al., 2016) and subtropical (Boddey et al., 2010; Albuquerque et al., 2015) regions of Brazil; however, they contrast with the results of studies conducted in temperate regions (Baker et al., 2007; Dimassi et al., 2014). This difference between regions can be due to additive factors, like differences in residue inputs (shoot and root), rainfall patterns (Hobley et al., 2016), and soil type (Torres-Sallan et al., 2017). In

European regions with rainfall levels varying between 350 and 800 mm year^{-1}, Dimassi et al. (2014) found a negative relationship between the increase in SOC stocks in NT vs. CT and annual precipitation. This precipitation level is well below the mean rainfall of the region where the present study was carried out (1440 mm year^{-1}). This higher rainfall, taken together with the more labile material left on the NT surface, may favor the percolation of dissolved organic carbon (COD) into the soil profile (Ota et al., 2013; Ono et al., 2015; Hobley et al., 2016).

In addition, the sandy texture of the soil in the superficial layer may have promoted rainwater percolation and, consequently, COD translocation into the soil profile (Wang et al., 2015). C sorption in minerals may be more efficient in the sub-superficial than in superficial layers because the higher C deficit saturation occurs in sub-superficial compared to superficial layer (Rumpel & Kogel-Knabner, 2011). This also shows the importance of cropping systems in terms of providing residues supporting for COD supply for the accumulation of SOC in deeper soil layers, especially in Acrisols, in which the clay content increases deeper in the soil profile, favoring C accumulation at depth (Torres-Sallan et al., 2017). The specific surface area and thus the adsorptive capacity of minerals in the subsoil are greater than in the superficial layer (Zinn et al., 2005). Furthermore, the oxide mineralogy of Acrisol is conducive to strong interactions with organic matter, leading to stabilization (Inda et al., 2007).

In humid subtropical climate conditions, for instance in southern Brazil, there is a greater potential for the distribution and accumulation of SOC along the soil profile. Considering the 0–100 cm soil depth, 30 years after the experiment began, high rates of C accumulation ranging from 0.38 to 1.15 Mg ha^{-1} year^{-1} were found (Table 9). Thus, our results may support country-specific SOC change factor in IPCC taking into account deeper layers, which maintain the SOC sequestration rate high after 30 years of conservation management.

The SOC sequestration data indicate that the soil can act as an important CO_2 sink in conjunction with agricultural activities, especially considering sequestration capacity at greater depths. The potential effect of NT associated with legume crops on SOC sequestration is an avenue that Brazil can pursue to meet its GHG emissions reduction target. After the Paris Agreement was reached at the Convention of the Parties (COP-21) in December 2015, the French government proposed the “4 per mille” initiative, which proposes soil

management options with a view to the sequestration and preservation of SOC (Minasny et al., 2017). Considering Brazil's mean SOC stocks of 45 Mg ha^{-1} (Fidalgo et al., 2007), its target under the 4 per mille initiative correspond to a SOC sequestration rate of 0.18 Mg ha^{-1} $year^{-1}$. This target has been shown to be attainable with the adoption of conservation management systems, such as the adoption of two legume cover crops in NT (0.76 Mg ha^{-1} $year^{-1}$). The results of this study show that, with proper agricultural practices, the carbon sequestration potential of Brazilian tropical and subtropical soils should be sufficient to reach or even exceed the target set out in the 4 per mille agreement (~ 0.6 Mg ha^{-1} $year^{-1}$).

The proposed conversion of 8 million hectares from CT to NT over the next few years in Brazil represents potential soil C storage of about 8 Tg C $year^{-1}$ (MAPA, 2012). Half of the area under NT in Brazil (16 Mha) is currently devoted to monocultures. Since the adoption of more diversified cropping systems could provide sequestration of 0.35 Mg ha^{-1} $year^{-1}$ (difference in SOC sequestration rate between NT O/M 180N and NT OV/MC 180N), the resulting improvement in the management system would result in additional SOC sequestration of 5.6 Tg $year^{-1}$. With the conversion of cropping areas to NT and the adoption of legume cover crops under NT, soil carbon sequestration capacity of 50 Tg CO_2 $year^{-1}$ could be attained in Brazil. If we consider the improvement of soil management systems alone in Brazil, it should be possible to reduce CO_2 emissions by 11%. This represents a considerable reduction in relation to the target set for Brazil 2025 (37% reduction by 2025) established at the Paris climate change conference.

5.6 Conclusion

Our study examined the effect of NT, legume cover crops, and nitrogen (N) fertilization from the perspective of potential SOC sequestration in superficial and sub-superficial soil layers. The results indicate that no-tillage with high and diversified residue inputs from legume cover crops is an effective long-term measure for soil carbon sequestration and mitigation of global warming in tropical and subtropical conditions. Although the rate of SOC accumulation in superficial layers decreases over time, conservation management systems favored SOC accumulation in sub-superficial layers of subtropical soils, helping to maintain high rates in the system after 30 years.

6. LONG-TERM LEGUME COVER CROPPING FAVOURS FORMATION OF MINERAL-ORGANIC ASSOCIATIONS ENRICHED WITH MICROBIAL METABOLITES

6.1 Abstract

The implementation of no-tillage (NT) coupled with legume cover cropping results in high carbon sequestration rates in soils of Southern Brazil. We hypothesized that this effect is due to the formation of mineral-organic associations (MOAs) through the enrichment in microbial metabolites, arising from the absence of tillage and the presence of high-quality legume residues. We sampled soil profiles in a long-term field experiment and used density and particle size fractionation in combination with carbohydrate and *n*-alkane analyses to compare the effect of conventional tillage (CT) vs. NT, combined or not with legume cover cropping, and combined or not with mineral N fertilization. While both NT and the presence of legume cover crops favoured the accumulation and enrichment in plant-derived carbohydrates in the surface soil layer (0-5 cm), due to the accumulation of plant residues, the ratio of microbe-derived carbohydrates (galactose, manose, fucose and rhamnose) to plant-derived carbohydrates (arabinose and xylose) increased with soil depth. In the 20–30 and 75–100 cm soil layers, the ratio of microbe-derived carbohydrates to plant-derived carbohydrates in the clay fraction was significantly greater with than without legume cover crops. These findings were in good agreement with the assessment of *n*-alkanes biomarkers: short *n*-alkanes chains (15–25 C atoms) were favoured by legumes under NT in the clay fraction of most soil layers, indicating predominance of microbial residues. This result suggests that the residue lability might be the main driver to C accumulation in MOAs. This study provides field-based evidence that the additional clay-bound organic C accumulating in the soil profile under NT with legume cover cropping is microbially processed.

6.2 Introduction

The Paris climate agreement aims at holding global warming below 2 ºC with efforts to limit it to 1.5 ºC (Rogelj et al., 2016). The soil organic carbon (SOC) pool might function as a significant sink for atmospheric carbon dioxide (CO_2) and contribute to mitigate anthropogenic greenhouse gas emissions, provided that soil be properly managed (Lal, 2004b). Changing agricultural practices represents one of the low-cost solutions proposed by Brazil to sequester carbon in soil and mitigate global warming (Brazil Ministry of the Environment, 2015).

Conversion from conventional tillage (CT) to NT is an important measure resulting in mean SOC sequestration rates of 0.32 and 0.48 Mg ha^{-1} $year^{-1}$ in tropical and subtropical regions, respectively, in the top 20 cm of soil (Bayer et al., 2006a). Recently, it was demonstrated that combining legume cover cropping with NT may result in SOC sequestration rates up to 1.15 Mg ha^{-1} $year^{-1}$ in subtropical soils down to 100 cm in the soil profile (Veloso et al., Submitted-b).

Previous studies have shown that MOAs are composed largely of microbial residues (Puget et al., 1998; Kogel-Knabner et al., 2008; Rumpel et al., 2010). Moreover, labile plant residues can induce microbial growth and have high microbial use efficiency, thereby favouring C accumulation through the formation of stable MOAs (Rumpel et al., 2010; Cotrufo et al., 2013). Formation of MOAs appears to be an important mechanism of C stabilization in tropical and subtropical soils as 65 to 92% of SOC in these soils is associated with kaolinite and Fe/Al oxides as the major clay constituents (Amado et al., 2006).

Carbohydrate and lipid analyses can be used as indicators to investigate the contribution of microbial products to SOC in MOAs. Carbohydrates containing the hexoses galactose (G), mannose (M),

fucose (F) and rhamnose (R) are considered to be synthesized predominantly by microorganisms, whereas carbohydrates containing the pentoses arabinose (A) and xylose (X) are synthesized predominantly by plants (Oades & Wagner, 1971; Cheshire, 1979). Thus, the GMFR/AX and GM/AX ratios have been used to estimate the relative contribution of microbial vs. plant derived carbohydrates in soils (Angers & Chantigny, 2008; Rumpel et al., 2010; Martins et al., 2012a). In addition to carbohydrates, lipids have also been used to infer the origin of SOC (van Bergen et al., 1998; Wiesenberg et al., 2004). Long-chain aliphatic structures of *n*-alkanes (25–35 C atoms) are predominantly derived from plants, whereas smaller chains are derived from microbes (van Bergen et al., 1998; Wiesenberg et al., 2004; Jandl et al., 2007) and the ratio [$\sum(C_{16}\text{-}C_{24}/\sum(C_{25}\text{-}C_{35})$] can be used as an indicator of the relative contribution of plants and microbes to SOC (Quénéa et al., 2006).

Our goal was to ascertain the theory that cropping systems with high residue lability associated with no-tillage (NT) favour the accumulation of microbial residues in MOAs and, thus, foster SOC accumulation. Using a long-term field experiment, we evaluated the effect of CT vs. NT, with or without legume cover cropping, and with or without mineral N fertilization, on carbohydrate and n-alkane and organic C (total and in MOAs) in surface and sub-surface soil layers of a subtropical Acrisol in Southern Brazil.

6.3 Materials and Methods

6.3.1 Description of the experiment

The study was conducted in a long-term experiment (30 years) located at the Agronomic Experimental Station of the Federal University of Rio Grande do Sul, in Eldorado do Sul–RS (30º06'S, 51º40'W and altitude of 96 m). The climate is subtropical (Cfa according to the Köppen classification), with mean annual temperature and annual rainfall of 19.4 °C and 1440 mm, respectively. The soil of the experiment is classified as

sandy clay loam granite-derived Acrisol (FAO, 2015). However, there is an increase in clay content down the soil profile, from 217 g kg^{-1} at 0–5 cm, 394 g kg^{-1} at 20–30 cm and reaching 511 g kg^{-1} at 75–100 cm. The main clay minerals are kaolinite (720 g kg^{-1}) and iron oxides (109 g kg^{-1}) (Bayer et al., 2001).

Until 1969, the area was under native grassland (mainly *Paspalum* and *Andropogon*). In the following 16 years, the soil was under intensive plowing and disking operations for annual cropping and was seriously degraded at the time of establishment of a factorial experiment in 1985.

The experimental treatments comprised two tillage practices, CT and NT, applied in main plots of 15 × 20 m, which were divided in three 5 × 20 m subplots to compare three crop succession: black oats (*Avena strigosa* Schreb) – maize (*Zea mays* L.) (O/M); vetch (*Vicia sativa* L.) – maize (V/M); oats + vetch – maize + cowpea (*Vigna unguiculata* (L.) Walp) (OV/MC). Each subplot of the six tillage-cropping combinations were divided in two 5 × 10 m sub-subplots receiving either 0 or 180 kg ha^{-1} of urea-N (0N and 180N), applied in strips in the maize crop. The experimental design was a split-split plot with three replicates arranged in randomized complete blocks.

Winter cover crops were sown in April-May, at a seed rate of 80 kg ha^{-1} for oats, 70 kg ha^{-1} for vetch, and 30+40 kg ha^{-1} for the oats+vetch combination. The cover crops were cut with a knife roller at the flowering stage (September-October). Tillage treatments and maize sowing were usually performed from 1 to 3 weeks later. In CT, operations included one plowing to 17-cm depth to incorporate cover crop residues, and two disking to 10 cm to prepare seedbed. In NT, soil was not disturbed, and crop residues left on the surface, except on plant rows for seeding. Maize was planted at a row-spacing of 90 cm, with a targeted density of 50–70 thousand plants per hectare. Fifty kg ha^{-1} of P_2O_5 and 50 kg ha^{-1} of K_2O were applied at planting. In OV/MC, cowpea was sown 20-30 days after maize planting, between maize rows at 40 cm between plants on the row.

After maize harvesting, cowpea reached full development and was terminated with a knife roller immediately before the establishment of oats and vetch.

Annual C inputs in the different management systems (aboveground plus root biomass, assuming root biomass equivalent to 30% of aboveground biomass) were compiled from 1985 to 2006 using data presented by Zanatta et al. (2007) , and updated until 2014.

6.3.2 Soil bulk density and SOC concentrations and stocks

Eight soil layers (0–5, 5–10, 10–15, 15–20, 20–30, 30–50, 50–75 and 75–100 cm) were sampled in September 2014, between winter cover crop termination and soil tillage for maize planting. Soil samples were collected with spiral auger (Φ= 20 cm) from all plots to determine SOC concentration. Soil samples were air dried and ground to ≤2 mm; a subsample of ~2 g was further ground to ≤250 µm in agate mortar for determination of SOC concentration by dry combustion (FlashEA 1112, Thermo Electron Corporation, Milan, Italy).

Trenches were opened in plots with a backhoe to measure soil bulk density in duplicate by the volumetric ring method (Blake & Hartge, 1986). Bulk density was measured in sub-subplots without nitrogen only, assuming that nitrogen fertilization did not modify soil density. The SOC stock in the 0-30 or 0-100 cm profiles was calculated based on the equivalent soil mass approach (Ellert & Bettany, 1995), which considers the mass of soil in all treatments to be similar to the mass of soil in a reference treatment, in this case CT O/M 0N. The annual SOC sequestration rate was calculated as the difference between the SOC stock of the treatments and that of the reference, divided by the 30-year duration of the experiment.

6.3.3 Soil organic matter fractionation and carbon concentration

The method described by Golchin et al. (1994) and adapted by Conceição et al. (2008) was used to determine the fractions of soil organic matter, where three fraction were obtained: free particulate organic matter, occluded particulate organic matter, and mineral-associated organic matter (min-OM). However, we used air-dried soil samples and not soil aggregates in this study because of we are working with whole-soil profile samples. Avoiding the underestimation of occluded-light fraction (Tomazi et al., 2011), we summed the free and occluded particulate organic matter fractions. Specifically, twenty grams of air-dried soil samples were placed in centrifuge tubes containing 70 mL of sodium polyntungstate solution (PTS) 2.0 g cm^{-3}. After slow and manual agitation to release the free light fraction without remove it, and the suspension was subjected to ultrasonic dispersion at the level of 240 J mL^{-1} to disperse soil aggregates and release the occluded light fraction (Conceição et al., 2008), and the sum of these formed the light fraction (LF). The level of energy for dispersion of the aggregates was previously defined in a specific test that proved sufficient to disperse microaggregates > 2 μm and recover 99% of the total clay fraction (Inda Junior et al., 2007). After dispersion of the sample, the suspension was centrifuged at 2000 g for 90 minutes. The supernatant floating LF was filtered (Whatman GF/C) under vacuum. The LF remaining on the filter was washed with distilled water and 0.01 mol L^{-1} calcium chloride solution ($CaCl_2$) to remove residual PTS. The rinsed LF was oven dried at 50 °C, ground manually in agate mortar, and analyzed for C content by dry combustion as described for SOC.

The sediment material containing the heavy fraction (HF) of SOC was then transferred and passed through a 53-μm mesh sieve to separate the sand-size HF fraction. The portion passed through the sieve (silt + clay) was transferred to a glass tube, and the volume was

completed to 1L with distilled water. At first, the clay fraction (Φ<0.002 mm) was separated according to Stokes' law, considering 25 ºC of temperature. The solution was mixed with a rod and, after 2 hours, 5 mL of the solution of the top 5 cm was collected, and then the water was completed to 1L again. This process was repeated 55 times to ensure that all the clay particles would be sampled. The material remaining in the test tubes, corresponding to the silt fraction, was also separated according to Stokes' Law in coarse silt (0.05-0.02 mm) and fine silt (0.02-0.002 mm) as described to clay-size HF fraction. The silt and clay fractions were flocculated using 2 M $CaCl_2$ solution, oven dried at 50 °C, weighed and milled in agate mortar. The C concentration in the sand-, coarse silt-, fine silt- and clay-sized HF fractions was determined by dry combustion as described earlier.

6.3.4 Soil Carbohydrates

In order to evaluate the effect of tillage, cropping systems, and nitrogen fertilization on the nature of SOC, carbohydrates and lipids analyses were carried out in whole soil and in the heavy fraction of SOC. As these methods are time consuming, analyses were made on three soil depths (0–5, 20–30 and 75–100 cm) in the following treatments: CT O/M 0N, NT O/M 0N, NT OV/MC 0N and NT OV/MC 180N. Analyses were carried out on soil samples collected on September 2014, prior to soil tillage and maize planting.

Carbohydrates were determined in whole soil and in the fine silt- and clay-sized HF fractions. Carbohydrates extractable with strong acid are considered to represent the total carbohydrates in the soil (Lowe, 1993). The method described by Chantigny & Angers (2008) was used for this determination. Specifically, 2 g of air-dried and finely ground (<0.15-mm) whole soil, and 1 g of fine silt and clay-sized HF fractions was weighed into a 50-mL centrifuge tube. Eight mL of 12 mol L^{-1} H_2SO_4 were added to whole soil and 4 mL to fine silt and clay fractions. The soil and acid

were gently mixed with a glass rod to ensure that the soil was completely moistened with the acid. The tubes were loosely capped and left on the bench for 2 h. After this period, the slurry was transferred into a 250-mL polypropylene bottle by washing the tube with 184 mL of deionized water to bring the solution to 0.5 mol L^{-1} H_2SO_4. The bottle was sealed and placed in an oven set at 85°C for 24 h. After cooling, the bottle was centrifuged at 16,000 × g for 10 min. A 15 mL aliquot of this supernatant was transferred to a plastic vial and stored at −20 °C for subsequent analysis.

Soil carbohydrates were analyzed based on the methods of Martens & Loeffelmann (2002) and Angers & Chantigny (2008). Briefly, 5 mL of soil hydrolysate was transferred in a 50-mL beaker and neutralized to pH 6.5-7.0 by slow addition of 0.5 mol L^{-1} NaOH solution. The volume of NaOH solution used was recorded to adjust calculation of carbohydrate concentrations. The neutralized solution was filtered using Whatman no. 42 filter paper. Approximately 2.5 mL of this filtrate was passed through a strong cation solid-phase exchange resin and collected in a 3-mL polypropylene Eppendorf vial. The cation-purified solution was then passed through a strong anion solid-phase exchange resin and collected in a new Eppendorf vial.

The monosaccharides present in the purified solutions were quantified using a liquid anion-exchange chromatograph with pulsed amperometric detection (Model Dionex ICS 5000+, Thermo Scientific, Sunnyvale, CA) equipped with automated diluter and sampler. The monosaccharide separation was performed with a CarboPac-PA1 pre-column (4 by 50 mm) in series with an analytical column (4 by 250 mm) and 10 to 50 µL injection loops. As eluents, we used 20 and 500 mmol L^{-1} NaOH solutions for monosaccharide analyses (0.8 mL min^{-1} flow rate) and conditioning of the column (1.0 mL min^{-1} flow rate), respectively. Monosaccharides were identified based on their retention time and coelution with spiked standards (Martens & Loeffelmann, 2002).

Blank samples were included during the extraction and purification processes to correct for the small amounts of arabinose, xylose, and glucose yielded by filter papers.

6.3.5 Soil lipids

Four grams of the clay-sized HF fraction were ground in agate mortar for free lipid extraction. The samples were Soxhlet-extracted with a dichloromethane–methanol (3:1 v/v) mixture for 24 h. The sequential elution was the hexane / toluene (6:4 v/v) mixture to separate aromatic hydrocarbons from *n*-alkanes. The fractions collected were reduced to a volume of approximately 1 mL with the aid of a rotary evaporator and transferred to chromatographic vials. Subsequently, the samples were dissolved in hexane, homogenized, and injected into a gas chromatograph coupled to a mass spectrometer (GC-MS) for *n*-alkanes determination. The analysis was performed with an HP5 MS fused silica capillary column (30 m × 0.25 mm Ø; 0.25-µm film thickness), and oven temperature programmed to increase from 40 °C to 290 °C at a rate of 6 °C min^{-1}. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1} , and the mass spectra were measured at 70 eV ionizing energy. Individual compounds were identified using low-resolution mass spectrometry and comparisons using published mass spectra libraries (NIST and Wiley). Traces corresponding to selected homologous series of lipid families were obtained using single-ion monitoring of ion characteristics such as ions at m/z 71 for *n*-alkanes.

6.3.6 Statistical analyses

After analyzed for normality by the Kolmogorov-Smirnov test and homogeneity of variance by the Levene test, the data were submitted to analysis of variance (ANOVA). The statistical model used to evaluate the effect of treatments on soil carbon and neutral sugar concentrations was: $Y_{ij} = \mu + B_i + T_j + Erro_{(ij)}$, where Y = mean of treatment j in block i; µ =

general average of the experiment; B = block (i = 1, 2, 3); T = treatments (j = 1, 2, 3, 4); Error = experimental error. All analyzes were performed using SAS ® v.9.4 (Statistical Analysis System Institute, Cary, North Carolina).

When significant ($p < 0.05$), treatment means were compared by contrast analysis using the Tukey's test ($p < 0.05$):

i) No-tillage effect: CT O/M 0N *vs*. NT O/M 0N;

ii) Legume effect: NT O/M 0N *vs.* NT OV/MC 0N and NT OV/MC 180N;

iii) Fertilization effect: NT OV/MC 0N *vs.* NT OV/MC 180N.

6.4 Results

6.4.1 Carbon input

The C input ranged from 4.8 to 10.2 Mg ha^{-1} $year^{-1}$ (Fig. 22). The C input was similar between CT and NT, but the inclusion of legume cover crops and N fertilization caused an increase of approximately 4 and 1 Mg ha^{-1} $year^{-1}$, respectively. Greater impact of N fertilization was observed on maize contribution to C annual addition, which increased from 38 to 51% in without and with N fertilization, respectively.

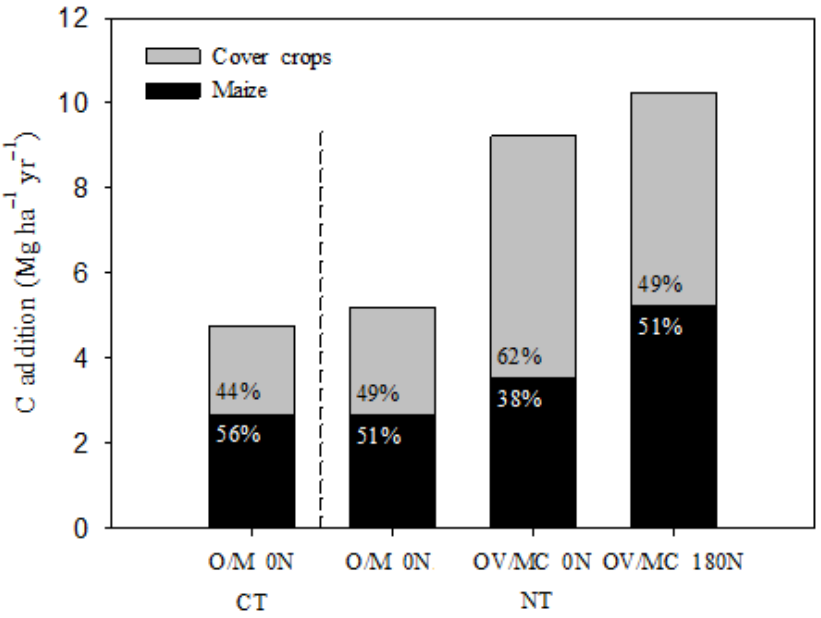

Figure 22. Mean annual C addition distribution across oat/maize (O/M) and oat + vetch/maize + cowpea (OV/MC) cropping systems subjected to two N-urea (0N = 0 kg ha^{-1} and 180N = 180 kg ha^{-1}).

6.4.2 *Stock and accumulation of soil organic carbon*

The SOC stock up to 100 cm depth ranged from 119.6 to 154.2 Mg ha^{-1} depending on the tillage and cropping practices (Table 11). The SOC stock increased 20 and 9 Mg ha^{-1} under NT and legume, respectively, compared to CT and without legume. The SOC accumulation ranged from 0.00 to 1.15 Mg ha^{-1} $year^{-1}$. SOC accumulation increased 0.66 Mg ha^{-1} $year^{-1}$ under NT as compared to CT. In NT, legume cover cropping favour greater SOC accumulation as compared to without legume.

Table 11. Soil organic carbon (SOC) stock and SOC accumulation up to 100 cm depth in a sandy clay loam Acrisol subjected to conventional tillage (CT) and no-till (NT) in combination to three cropping succession: oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC); and two N-urea rates (0 = 0 kg ha^{-1} and 180 = 180 kg ha^{-1}).

Tillage	Crop succession	N fertilization	SOC stock	SOC accumulation
			Mg ha^{-1}	Mg ha^{-1} yr^{-1}
CT	O/M	0	119.59	0.00
NT	O/M	0	139.46	0.66
	OV/MC	0	148.19	0.95
	OV/MC	180	154.20	1.15
Contrast	No-tillage		-19.87*	-0.66*
	Legume		-23.46*	-0.78*
	Fertilization		-6.01	-0.20

Contrast analysis was used to test the effects of no-tillage (CT O/M 0 vs. NT O/M 0), legume cover cropping (CT O/M 0 vs. NT OV/MC 0 + NT OV/MC 180) and nitrogen fertilization (NT OV/MC 0 vs. NT OV/MC 180). Significant differences between treatments at 5% and 1% probability of error by F test are designed with (*) and (**), respectively.

6.4.3 Organic carbon in whole-soil and fractions

The SOC concentration ranged from 10.6 to 25.9 g kg^{-1} in the 0–5 cm soil layer and was greater under NT, legume and N fertilization compared to CT, without legume and without N (Table 12). In 20–30 cm soil layer, there was a greater SOC concentration under legume than without legume, while SOC concentration decreased with as compared to without N fertilization. The SOC concentration ranged from 5.4 to 6.7 g kg^{-1} in the 75–100 cm soil layer and was greater under NT, compared to CT.

The LF-C concentration ranged from 1.5 to 9.2 g kg^{-1} in the 0–5 cm soil layer depending on management system (Table 12). In this surface soil layer, LF-C doubled under NT as compared to CT. In NT systems,

legume cover cropping doubled LF-C concentration and N fertilization tripled concentration as compared to without legume and N. The concentration of LF-C increased more rapidly than total SOC as a function of treatments and accounted for 14% of SOC under the CT-cereal system, 21% in NT-cereal system, 29% in NT-cereal-legume system, and 35% in NT-cereal-legume-N system. In deeper soil layers, LF-C represented less than 3.2% of SOC, and was not different among management systems.

The content of sand-C and coarse silt-C represented a small proportion of whole-soil SOC (<8%) and were not different among management practices at any soil depths (Table 12). The fine silt-C concentration ranged from 9 to 29.6 g kg^{-1} in the surface layer and was twice as high under NT as under CT, and was also increased by the inclusion of legumes in the crop rotation. The fine-silt-C fraction accounted for 14 (CT-cereal system) to 22% (NT-cereal system) of SOC. Fine silt-C concentration was not different among management systems in deeper soil layers.

The clay-C concentration was affected by management practices in different soil layers. In the surface layer, clay-C was not influenced by tillage, but increased from 7.1 g kg^{-1} to 9.6 g kg^{-1} on average with the inclusion of legumes in cropping practice (Table 12). In the 20–30 and 75–100 cm soil layers, clay-C was mainly affected by tillage systems ($p<0.01$), with greater concentration in NT compared to CT (Table 12). The clay-C fraction always accounted for the greatest portion of total SOC, especially in deeper soil layers (85% on average in the 20–30 and 75–100 cm layers). This was also true for the surface soil layer, but values differed among management systems and were at the reverse of LF-C and fine silt-C fractions, with 63% in CT-cereal system, 47% in NT-cereal system, 44% in NT-cereal-legume system, and 39% in NT-cereal-legume-N system.

Table 12. Carbon concentration in light (LF-C) and heavy fractions (C associated with sand, coarse silt, fine silt and clay fractions) of soil organic matter, and its proportion to total soil organic carbon (SOC) in the 0–5, 20–30 and 75–100 cm layers in an Acrisol submitted to conventional tillage (CT) and no tillage (NT), with oat/maize (O/M) or oat+vetch/maize+cowpea (OV/MC) succession, and with (180 kg urea-N ha^{-1} [180N]) or without (0N) nitrogen fertilization.

Tillage	Crop succession	Fertilization	SOC	LF-C	Sand-C	Coarse silt-	Fine silt-C	Clay-C
			---------- g kg^{-1} soil----------					
						0-5 cm		
CT	O/M	0	10.6	1.49	0.25	0.67	1.61	6.53
NT	O/M	0	15.0	3.18	0.25	1.23	3.24	7.07
	OV/MC	0	20.9	5.99	0.24	1.43	3.97	9.24
	OV/MC	180	25.9	9.16	0.29	1.83	4.61	10.01
Contrast†	No-tillage		-4.4*	-1.69#	0.01	-0.56	-1,63 *	-0.54
	Legumes		-16.8*	-8.79**	0.03	-0.80	-2.09*	-5.11*
	Fertilization		-5.0*	-3.17 *	-0.05	-0.39	-0.64	-0.77
						20 – 30 cm		
CT	O/M	0	9.1	0.23	0.16	0.36	0.94	7.42
NT	O/M	0	9.4	0.29	0.13	0.38	0.90	8.25
	OV/MC	0	10.6	0.32	0.17	0.31	1.02	8.77
	OV/MC	180	10.0	0.31	0.12	0.27	0.96	8.36
Contrast	No-tillage		-0.3	-0.06	0.03	0.03	-0.04	-0.83**
	Legumes		-1.8#	-0.04	-0.03	0.18	-0.18	-0.64#
	Fertilization		0.6#	-0.01	0.01	0.11	-0.06	0.11#
						75 – 100 cm		
CT	O/M	0	5.4	0.06	0.09	0.18	0.42	4.70
NT	O/M	0	6.5	0.04	0.10	0.20	0.51	5.68
	OV/MC	0	6.7	0.07	0.09	0.24	0.50	5.82
	OV/MC	180	6.4	0.07	0.09	0.23	0.49	5.85
Contrast	No-tillage		-0.9*	0.02	-0.02	-0.02	-0.08	-0.98*
	Legumes		-0.1	-0.05	0.03	-0.07	0.03	-0.32
	Fertilization		0.3	0.01	0.01	0.01	0.01	-0.03

†Contrast analysis was used to test the effects of no-tillage (CT O/M 0 vs. NT O/M 0), legume cover cropping (CT O/M 0 vs. NT OV/MC 0 + NT OV/MC 180), and nitrogen fertilization (NT OV/MC 0 vs. NT OV/MC 180). Significant differences between treatments at 10%, 5%, 1% and <1 % probability of error by F test are designed with (#), (*), (**) and (***), respectively.

6.4.4 Neutral carbohydrates

Plant-derived carbohydrates (AX values in Table 13) ranged from 658 to 2096 mg kg^{-1} in whole soil and fractions, and markedly declined down the soil profile. In the surface soil layer, the concentration of plant-derived carbohydrates was 1.5 times greater under NT than CT in the fine silt fraction and 1.3 times greater under NT in the clay fraction; the same trend was found in whole soil (1.5 times greater concentration under NT than CT). Plant-derived carbohydrates were also 1.8 times more abundant, on average, in whole soil where legumes were included in the crop rotation and 1.4 times more abundant with than without N fertilization. Similar effects of legumes and fertilization were also found in the clay and fine-silt fractions. A positive effect of legumes was also found in the 20–30 and 75–100 cm depths, in whole soil and clay fraction, whereas a negative effect of NT was found at the 75-100 cm depth in the clay fraction. As compared to whole soil, similar concentrations of plant carbohydrates were found in the fine silt and clay fractions of the surface soil layer. In the sub-surface layers, the concentrations were greater in the clay fraction, whereas the fine silt fraction had concentration similar or lower than the whole soil.

Table 13. Total plant- (AX) and microbe-derived (GMRF) carbohydrates and GMFR/AX ratio in whole soil (WS) and clay- and fine silt-sized fractions in 0–5, 20–30 and 75–100 cm layers of an Acrisol submitted to conventional tillage (CT) and no tillage (NT), with oat/maize (O/M) or oat+vetch/maize+cowpea (OV/MC) succession, and with (180 kg of urea ha^{-1} [180N]) or without (0N) fertilization.

Tillage	Crop	Fertilization	AX‡			GMFR‡			GMRF/AX ratio		
			WS	Clay	FS	WS	Clay	FS	WS	Clay	FS
			mg kg^{-1}								
			0-5 cm								
CT	O/M	0	657.6	988.8	709.6	880.1	1813.2	981.	1.39	1.83	1.38
NT	O/M	0	1015.4	1283.3	1628.1	1156.5	2443.2	244	1.13	1.90	1.50
	OV/MC	0	1541.2	1744.1	1833.8	1506.5	3100.4	265	0.98	1.78	1.45
	OV/MC	180	2096.0	1875.7	2059.0	1880.5	3398.2	294	0.90	1.81	1.43
Contrast†	No-tillage		-357.8#	-294.5**	-918.5***	-276.4#	-629.9**	-1463.7***	0.25	-0.07	0.13
	Legumes		-1074.1**	-1612.3**	-714.3**	-1606.5**	-1053.2***	-636.6**	0.38*	0.22*	0.13
	Fertilization		-554.8*	-131.6*	-225.3**	-374.0*	-297.8*	-294.9*	0.08	-0.04	-0.01
			20 – 30 cm								
CT	O/M	0	421.0	690.9	416.4	1051.6	1317.6	647.	2.44	1.92	1.52
NT	O/M	0	473.2	891.7	413.1	909.6	1686.7	667.	1.93	1.89	1.60
	OV/MC	0	506.0	917.2	438.0	1184.9	1845.4	683.	2.33	2.05	1.55
	OV/MC	180	491.2	805.9	347.1	972.4	1551.7	553.	1.98	2.02	1.54
Contrast	No-tillage		-52.2	-200.8	6.3	141.7	-369.1*	-	0.51	0.03	-
	Legumes		-	-23.7	98.9	-51.1	60.4	41.4	-0.46	-	0.12
	Fertilization		15.2	111.3	90.9	212.6	293.6*	129.	0.34	0.02	0.04
			75 – 100 cm								
CT	O/M	0	282.6	393.4	125.2	704.7	823.9	149.	2.65	2.17	1.11
NT	O/M	0	262.3	351.6	112.7	575.0	666.5	130.	2.28	2.01	1.16
	OV/MC	0	303.4	484.1	159.7	767.4	1034.3	180.	2.85	2.17	1.12
	OV/MC	180	369.5	476.9	138.6	853.9	1008.6	150.	2.49	2.12	1.09
Contrast	No-tillage		20.4	41.8**	12.5	130.2*	157.3**	18.7	0.37	0.17	-0.14
	Legumes		-471.8	-709.9***	-69.6	-148.3**	-257.9*	-	-0.79	-0.27#	0.11
	Fertilization		-66.1	7.2	21.3	-86.5*	25.8	30.1	0.36	0.06	-0.04

†Contrast analysis was used to test the effects of no-tillage (CT O/M 0 vs. NT O/M 0), legume cover cropping (CT O/M 0 vs. NT OV/MC 0 + NT OV/MC 180) and nitrogen fertilization (NT OV/MC 0 vs. NT OV/MC 180). Significant differences between treatments at 10%, 5%, 1% and <1 % probability of error by F test are designed with (#), (*), (**) and (***), respectively.

‡ AX, sum of arabinose and xylose concentrations; GMFR, sum of galactose, mannose, fucose and rhamnose concentrations.

Microbe-derived carbohydrates (GMFR values in Table 13) ranged from 880 to 3398 mg kg^{-1} in whole soil and fraction in the surface soil layer and declined more gradually than plant-derived carbohydrates down the soil profile, with values ranging from 554 to 1845 mg kg^{-1} at 20–30 cm depth, and from 131 to 1034 mg kg^{-1} at 75–100 cm depth. In surface soil layer, microbe-derived carbohydrates were more abundant under NT than CT in the fine silt and clay fractions; a similar trend was found for whole soil. Microbe-derived carbohydrates were also more abundant in the presence of legumes and with N fertilization in whole soil and fractions of the surface soil layer, and treatment effects were more obvious in fractions than in whole soil. A positive effect of NT and a negative effect of fertilization were found in the 20–30 cm soil layer in the clay fraction only. A negative effect of NT was found in whole soil and clay fraction at the 75–100 cm depth, whereas a positive effect of legumes was found in whole soil and both fractions. As compared to whole soil, the clay fraction was enriched in microbe-derived carbohydrates in all soil layers. In contrast, the fine silt fraction was enriched in microbial carbohydrates in the surface soil layer compared to whole soil but was impoverished at greater depths.

The microbe-to-plant-derived carbohydrate ratio (GMFR/AX values in Table 13) ranged from 0.90 to 2.85 and was generally narrower in surface than in deeper soil layers. In the whole soil and clay fraction of surface soil layer, the ratio was narrower with than without legumes. In contrast, the ratio was significantly wider with than without legume cover crops in clay fraction of 20–30 cm soil layer, and in wholes soil and clay fraction of the 75–100 cm soil layer. Tillage and N fertilization had no effect on the microbe-to-plant-derived carbohydrate ratio.

6.4.5 Free Lipids

The ratio of short (C_{16}-C_{25}) to long chain (C_{26}-C_{35}) n-alkanes ($R_{S/L}$) ranged from 0.34 to 159 (Table 14). Wider values were found in the clay fraction and indicated the predominance of small chain *n*-alkanes ($R_{S/L}$ >1) compared to whole soil in the surface soil layer; predominance of small chain *n*-alkanes was also generally found in the clay fraction at greater depths. The $R_{S/L}$ values were

generally similar between CT and NT. In clay fraction of, the $R_{S/L}$ ratio was wider with than without legume cover crops in the 0–5 and 20–30 cm soil layers. Management practices did not influence $R_{S/L}$ in the 75–100 cm soil layer.

Table 14. Ratios of short chain-to-long chain *n*-alkanes in whole soil and the clay fraction of an Acrisol under conventional (CT) or no-tillage (NT), with oat/maize (O/M) or oat+vetch/maize+cowpea (OV/MC) succession, and with (180 kg urea-N ha^{-1}) or without fertilization.

Tillage	Crop succession	Fertilization	Whole soil Rs/l	Clay Rs/l
		0 – 5 cm		
CT	O/M	0	1.01	2.61
NT	O/M	0	0.73	2.99
	OV/MC	0	0.89	6.76
	OV/MC	180	0.34	4.19
		20 – 30 cm		
CT	O/M	0	nd	3.43
NT	O/M	0	nd	0.73
	OV/MC	0	nd	159.43
	OV/MC	180	nd	50.43
		75 – 100		
CT	O/M	0	nd	-
NT	O/M	0	nd	2.90
	OV/MC	0	nd	1.56
	OV/MC	180	nd	2.98

[1]Rs/l, ratio between short and long chain *n*-alkanes ($\Sigma C_{16-25}/\Sigma C_{26-35}$); nd = not determined.

6.5 Discussion

The greater SOC concentration under NT than CT in the surface soil layer was explained mainly by increases in LF-C and fine silt-C fractions. It is suggested that, compared to CT, reduced soil disruption under NT favoured the accumulation of plant residues at the soil surface (greater LF-C concentration) and its occlusion within aggregates (Conceição et al., 2013; Veloso et al., Submittted-a) where it became integrated in silt-sized MOAs. Soil aggregates under NT were proposed

as microsites where decomposing organic residues SOC formation and stabilization occur through the formation of MOAs (Conceição et al., 2013). This is supported by the much greater amount of fine-silt C found under NT compared to CT in the surface soil layer. The positive effect of NT on SOC accumulation was more visible in the clay fraction in the 20–30 and 75–100 cm soil layers. That SOC accumulation was more directly associated with the clay fraction in deeper soil layers might be explained by the presence of biopores that possibly favoured the percolation of soluble C compounds down the soil profile, which were adsorbed by fine mineral particles (Hobley et al., 2016).

The generally greater SOC concentration with than without legume cover crops was mainly explained by greater C concentrations in LF and clay fractions in the surface soil layer. Consequently, both NT and the presence of legume cover crops favoured the concentration of plant residues (more LF-C). In contrast, C concentrated in different types of MOAs depending on management practice: while tillage influenced C concentration in the fine silt fraction only, legume cover cropping increased C concentration in fine silt and clay fractions. This may be related to the fact that tillage affects the distribution of crop residues within the soil and stimulates their initial decomposition and formation of coarse MOAs by increasing contact with mineral particles (Reis et al., 2014); in contrast, the presence of legumes may have a more direct impact on the formation of clay-size MOAs by stimulating microbial growth through low C/N substrates (Cotrufo et al., 2015). The C in the clay fraction favoured by two legume cropping system compared to with no legumes cropping system under NT led to SOC accumulation in whole soil profile which resulted in C sequestration rates higher than 0.9 Mg ha^{-1} $year^{-1}$ (Table 11).

As compared to whole soil, the clay fraction was generally enriched in plant- and microbe-derived carbohydrates, and is generally attributed to combined microbial colonization, decomposition, and encrustation of plant residues leading to the formation of stable organic matter in clay-sized MOAs. The exception noticed for plant carbohydrates in the surface soil layer is likely the result of the large abundance of LF-C in the surface soil layer that led to an overwhelming

contribution of plant-derived carbohydrates. This is supported by the fact that plant-derived carbohydrate concentrations were the closest between whole soil and fractions in systems with legume cover cropping and N fertilization where LF-C concentrations were the highest. Abundant LF-C in surface soil also explains the relatively narrow microbe-to-plant-derived carbohydrate ratio found in whole-soil, especially with legumes and N fertilization.

The widening of microbe-to-plant carbohydrate ratio between whole soil, fine silt, and clay fractions in the surface soil layer suggests that plant residues in whole soil (mainly LF-C) were gradually converted to smaller plant residues partly colonized by microbes and partly encrusted with minerals (fine silt-sized MOAs), and then to more thoroughly processed materials enriched in microbial metabolites (clay-sized MOAs). The enrichment in microbial carbohydrates and differences between whole soil and the clay fraction were markedly amplified by legume cover cropping (NT O/M 0N *vs*. NT OV/MC 0N). This is in good agreement with the marked widening of short-to-long *n*-alkanes chains under legume cover cropping in the clay fraction, indicating strong predominance of microbial residues in the presence of legume crops. These findings provide field-based evidence that high-quality legume residues favour the accumulation of carbon in fine MOAs, as compared to lower quality residues from maize and cereals, by favouring microbial processing and accumulation of microbial metabolites. These field-based results thus support the Microbial Efficiency-Mineral Stabilization theory (Cotrufo et al. 2013).

The positive effect of legume cover cropping on enrichment in microbe carbohydrates was also observed in clay fraction of the 20–30 cm and 75–100 cm soil layers. This suggests that legumes also stimulated accumulation of microbial metabolites at depth and formation of fine MOAs, but that this effect was mainly mediated by the rooting systems through root decay and/or exudation.

The positive effect of high-quality legume residues on accumulation of microbial residues and formation of MOAs was further confirmed by the lack of effect of nitrogen fertilization on carbohydrate and *n*-alkanes ratios in the clay fraction. Compared to no fertilization, carbon return to soil with maize residues was

increased by N fertilization (Fig. 22). This resulted in greater LF-C concentration, but did not change C concentration in the fine silt and clay fractions, compared with no fertilization. In addition, the relative abundance of microbial-derived short chain n-alkanes was greater with than without N in the 0–5 and 20–30 cm soil layers. This is likely due to lower concentrations of N and soluble organic compounds in maize residues than in legume residues (Pimentel el al., 2015). In addition, the greater lignin concentration in maize than legume residues likely favoured the physical transfer of residues to soil as LF-C but not the stabilization in MOAs (Spielvogel et al., 2008; Cotrufo et al., 2015). Our results also agree with Yu et al. (2016) that found lower microbial biomass with than without N fertilization.

Whole soil was enriched in microbial carbohydrates in sub-surface layers whereas LF-C concentrations were one to two orders of magnitude lower than in the surface soil layer. Further to earlier discussion, changes in soil C and carbohydrate profile at depth were likely meditated by stimulation of soil microbes through root decay and exudation and accumulation. However, in contrast with surface soil, the microbe-to-plant carbohydrate ratio was generally wider in whole soil than in the fine silt and clay fractions. This indicates that preferential accumulation of microbial metabolites at depth did not necessarily lead to accumulation in MOAs in the bulk soil but may have been limited to rhizosphere, root channels and other macropores with little interaction with the bulk soil mineral matrix as previously proposed for accumulation of C in forest soil profiles (e.g., Guggenberger & Kaiser (2003)).

6.6 Conclusions

Using a long-term field experiment, we found that tillage influenced C concentration in the fine silt fraction only, while legume cover cropping increased C concentration in fine silt and clay fractions. Our results suggest that fine silt MOAs is an intermediate between young organic matter (LF) and stabilized OM in clay MOAs. Our results confirmed the hypothesis that carbon present in mineral-organic associations is dominated by microbial-derived residues. The legume cover crops under NT further favour the dominance of microbial over plant-derived

residues in fine fractions. The addition of residues enriched in nitrogen and soluble fraction through legume cover crops are the main driver to C accumulation in mineral-organic association. These results were verified in whole soil profile (100 cm soil depth) and support the high carbon accumulation obtained in subtropical region with the adoption of no-tillage and legume cover crops.

7. SOIL AGGREGATION AND CARBON ACCUMULATION ARE MEDIATED BY FUNGI IN A SUBTROPICAL SOIL UNDER CONSERVATION AGRICULTURE

7.1 Abstract

No-tillage (NT) and legume cove crops have been found to improve the quality of tropical and subtropical soils. Microbial cell-wall constituents have been evidenced as important contributors to soil aggregation and soil organic carbon (SOC) formation. We performed a study to investigate the influence of NT and legume cover crops on the microbial cell-wall constituents [glucosamine (GlcN), taken as indicator of fungal cell-wall constituents; muramic acid (MurN), taken as indicator of bacterial cell-wall constituents] and their potential influence on soil aggregation and SOC accumulation in a subtropical Acrisol in Southern Brazil. The GlcN content ranged from 450.5 mg kg^{-1} in the 0–5 cm soil layer to 20.5 in the 75–100 cm soil layer, approximately 10 times the range of MurN contents, from 53.1 to 2.7 mg kg^{-1} for the same soil layers. NT and legume cover crops favoured the accumulation of fungal and bacterial cell-wall constituents in whole soil, especially in the top 5 cm of soil with a preferential enrichment of soil organic C in GlcN. In that soil layer, the GlucN/MurN ratio was strongly and positively correlated with the content of light-fraction organic matter (LF) and mean-weight diameter of soil aggregates. Fungal-derived glucosamine also preferentially accumulated down to 100 cm depth, and more specifically in the clay-sized fraction of soil, suggesting a specific role in SOC accumulation at depth. In the surface soil, the greater LF content under NT and legume cover crops favoured the fungal community which, in turn, mediated the improvement in soil aggregation. Overall, our study provides field-based evidence that the accrual of fungal cell-wall constituents is a key process leading to SOC accumulation and soil aggregation under NT and legume cover crops in subtropical soils.

7.2 Introduction

Adoption of conservation practices is essential to maintain or improve crop production and the sustainability of agricultural systems, especially in tropical and subtropical environments where SOC has a central role in soil functioning (Lal, 2009; Powlson et al., 2016), and where its decline has been observed following intensive cultivation (Zanatta et al., 2007).

No-tillage (NT) agriculture is practiced on about half of the agricultural land of Brazil (FEBRAPDP, 2012) and generally favours SOC accumulation compared to conventional tillage systems (Bayer et al., 2006a). The effect of NT on SOC is closely linked to soil macroaggregation through occlusion mechanisms (Veloso et al., Submitted-a). When coupled with NT, cover cropping with legumes has also been shown to favour SOC accumulation and soil aggregation under tropical/subtropical conditions (Conceição et al., 2013).

Changes in soil microbial communities, including increases in bacterial and fungal biomass, have been observed after conversion from CT to NT in temperate environments (Minoshima et al., 2007). Soil microbes, especially fungi, have long been recognized as playing a significant role in the formation and stabilization of soil aggregates (Chantigny et al., 1997). Moreover, fungi contribute more to the formation of aggregates in NT than CT soils (Beare et al., 1992; Helgason et al., 2010). Fungal hyphae increase the resistance of aggregates to mechanical breakdown and resistance to the action of water (Abiven et al., 2007).

Recent models of SOC formation now consider microbial residues as the main source for stable soil organic matter (Kogel-Knabner, 2002). Moreover, SOC stabilization by the soil matrix and consequent soil aggregation would be favoured by more labile C inputs (Cotrufo et al., 2015). More field-based observations are needed to support these concepts. Cell-wall constituents of fungi and bacteria in soil can provide the “fingerprint” for SOC formation (Ding et al., 2011). Contents of muramic acid (MurN) and glucosamine (GlcN) have been used to distinguish respectively bacterial and fungal cell wall residues, mainly in temperate environments (Chantigny et al., 1997; Amelung et al., 2008). Only recently few

studies have been performed in subtropical/tropical soils where a favorable effect of NT in total amino-sugar with preferential glucosamine enrichment in comparison to conventionally-tilled soil (Ding et al., 2011) and a co-accumulation in similar proportion of both MurN and GlcN by the use of legume cover crops has been noticed (Martins et al., 2012b).

Thus, the literature available on the influence of conservation practices on soil bacterial and fungal constituents, in relation to soil aggregation and SOC dynamics, is scant for subtropical and tropical conditions. We performed a study to investigate the influence of NT and legume cover crops on microbial cell wall constituents in a subtropical Acrisol in Southern Brazil, and examined the relationships with soil aggregation and accumulation of organic C in whole soil and SOC fractions.

7.3 Materials and Methods

7.3.1 Description of the experiment

The study was conducted in a 30-years old experiment located at the Agronomic Experimental Station of the Federal University of Rio Grande do Sul, in Eldorado do Sul, Brazil (30º06'S, 51º40'W; altitude 96 m asl). The climate is subtropical (Cfa according to the Köppen classification), with mean annual temperature and annual rainfall of 19.4 °C and 1440 mm, respectively. The soil of the experiment was classified as sandy clay loam, granite-derived Acrisol (FAO, 2015). However, the clay content increases from 217 g kg^{-1} at 0–5 cm to 511 g kg^{-1} at 75–100 cm depth. The main clay minerals are kaolinite (720 g kg^{-1} clay) and iron oxides (109 g kg^{-1} clay) (Bayer et al., 2001).

Until 1969, the area was under native grassland (mainly *Paspalum* and *Andropogon*). In the following 16 years, the soil was seriously degraded by intensive plowing and disking operations for annual cropping, until the establishment of the experiment in 1985.

The experimental treatments comprised two tillage systems, CT, reduced tillage and NT applied in main plots of 15 × 20 m, and combined with three cropping systems applied in subplots of 5 × 20 m in size. The cropping systems were: black

oats (*Avena strigosa* Schreb) / maize (*Zea mays* L.) (O/M), vetch (*Vicia sativa* L.) / maize (V/M), and oats + vetch / maize + cowpea (*Vigna unguiculata* (L.) Walp) (OV/MC). Each of the nine tillage-cropping combinations was submitted to two fertilization levels: 0 and 180 kg urea-N ha^{-1} (0N and 180N), banded in the maize crop, in sub-subplots of 5 × 10 m. The experimental treatments were arranged in a split-split plot design with three replicates. More details are provided in Bayer et al. (2000) and Zanatta et al. (2007).

Trenches were opened with a backhoe and eight soil layers (0–5, 5–10, 10–15, 15–20, 20–30, 30–50, 50–75 and 75–100 cm) were sampled in September 2014, prior to soil tillage and maize planting. Four treatments were selected (CT O/M 0N, NT O/M 0N, NT OV/MC 0N and NT OV/MC 180N) for the present study. Soil samples were air dried and ground to ≤2 mm; and a subsample of ~2 g was further ground to ≤250 μm for determination of accumulation of organic C via dry combustion (FlashEA 1112, Thermo Electron Corporation, Milan, Italy) and amino-sugars in whole soil and SOC fractions.

Annual carbon inputs in the cropping systems were calculated using a database compiling information since inception of the experiment in 1985 (Zanatta et al., 2007), and updated until year 2014 (root C input was estimated to be 30% of C input from aboveground parts) (Table 15).

Table 15. Soil characteristics of the long-term field experiment (30 years) used in this study, comparing conventional tillage (CT) and no-tillage (NT) combined with three cropping systems: (i) black oat (Avena strigosa Schreb) as winter cover crop followed by maize (Zea mays L.) as summer grain crop (O/M) and (ii) oat plus vetch (Vicia sativa) followed by maize in summer intercropped with cowpea cover crop (Vigna unguiculata (L.) Walp) (OV/MC).

Characteristic		CT	NT		
		----	----------		
		O/M 0N	O/M 0N	OV/MC 0N	OV/MC 180N
C addition (Mg ha^{-1} yr^{-1})[2]		4.8	5.2	9.2	10.2
Soil carbon (g kg^{-1})[2]					
0–5 cm		10.6	15.0	20.9	25.9
20–30 cm		9.1	10.0	10.6	10.0
75–100 cm		5.4	6.5	6.7	6.7
Light fraction-C (g kg^{-1})[3]					
0–5 cm		1.5	3.2	6.0	9.2
20–30 cm		0.2	0.3	0.3	0.3
75–100 cm		0.1	0.1	0.1	0.1
Clay-C (g kg^{-1})[3]					
0–5 cm		6.5	7.1	9.2	10.0
20–30 cm		7.4	8.3	8.8	8.4
75–100 cm		4.7	5.7	5.8	5.9
Aggregate distribution (%)[2]					
0–5 cm	> 2 mm	12.5	29.2	37.3	-
	2 – 0.25 mm	52.6	45.1	42.3	-
	< 0.25 mm	34.9	23.7	20.4	-

[2] original data presented by Veloso et al. (Submitted a); [3] Original data presented in Chapter 5.

7.3.2 Amino-sugar determination

Amino-sugars were measured in whole soil, and in fine-silt and clay sized fractions. The soil fractions were obtained through a fractionation scheme detailed in Chapter 5 of this thesis. Briefly, the light fraction (LF) was separated using a sodium polytungstate (NaW) suspension after dispersion of soil by sonication. The

floating LF was removed using vacuum and washed with water and calcium chloride 0.01 mol L^{-1} to remove NaW. The material was dried (50°C, 48 h) and weighed to determine dry matter content, and analyzed for C content by dry combustion. The suspension was then passed through a sieve of 53 µm in order to separate the sand fraction. The portion passed through the sieve was transferred to a graduated cylinder (1L) where 55 samples of the clay fraction (<0.002 mm) were made, based on Stokes' Law, until its complete removal. The material remaining in the graduated cylinder, corresponding to the silt fraction, was also separated according to Stokes' Law in coarse-silt (0.05-0.02 mm) and fine-silt (0.02-0.002 mm). The corse silt, fine silt and clay fractions were flocculated with $CaCl_2$ 2 mol L^{-1} , and then oven dried at 50 ° C, weighed and grounded in agate mortar.

Amino-sugar content was determined by the method of Angers & Chantigny (2008), as modified from (Zelles, 1988). Briefly, 1 g of air-dried and finely ground (<0.15 mm) soil was transferred to 30-mL glass test tubes, and 20 mL of 6 mol L^{-1} HCl was added to the tubes and mixed thoroughly on a vortex. The mixture was carefully bubbled with N_2 to remove O_2 and minimize amino-sugar oxidation. Tubes were sealed with screw caps, incubated for 6 h in an oven set at 105 ºC, then cooled on ice and centrifuged at 1500×g for 10 min. A 1-mL aliquot of the supernatant was transferred to a 25-mL boiling flask and evaporated to dryness with a rotary vacuum evaporator. The precipitate was dissolved in 980 µL of O-phthaldehyde (5 g OPA L^{-1} dissolved in 0.5 mol L^{-1} potassium tetraborate solution), and 20 µL of 2-mercaptoethanol was added to the flask, starting the derivatization. The mixture was vortexed, transferred to a 1.5 mL Eppendorf tube, and centrifuged at 20,000×g for 3 min. Five minutes (±15 s) after 2-mercaptoethanol addition, a 25-µL aliquot of the supernatant was injected in a high-performance liquid chromatograph (Waters, Milford, MA, USA) was equipped with a pump (model 515, Waters) and an analytical column (4 mm, 8×100 mm, Nova-Pak C18, Waters) maintained at 40 ºC. The eluents used were: solution A (0.05 mol L^{-1} sodium citrate; 0.05 mol L^{-1} sodium acetate; methanol : tetrahydrofuran, in proportion 18 : 1.7 : 1) and solution B (65% methanol) at a flow

rate of 1.5 mL min^{-1}. The proportion of solution A (gradual change) was: 85%, 0 to 2.5 min; 70%, 2.5 to 17.5 min; 85% 17.5 to 30 min. Detection was performed by fluorescence (scanning fluorescence detector, model 474, Waters) with an emission wavelength of 425 nm and an excitation wavelength of 338 nm using a Xenon lamp. Amino-sugars from soil samples were identified and quantified by comparison to chromatograms of standard solutions containing mixed MurN and GlcN. Amounts of C in amino-sugars were calculated considering that MurN contains 43% C and GlcN contains 40% C.

Total MurN-C in the soil was taken as a proxy for total bacterial cell-wall mass as bacterial cells are considered the exclusive source of MurN in soil (Zelles, 1988; Angers & Chantigny, 2008). Bacteria cell walls also contain GlcN in a ratio of 2:3 with MurN, on a C basis. We therefore subtracted 0.67 times MurN-C from soil total GlcN-C to estimate fungal-derived GlcN content (Chantigny et al., 1997). Potential error in this estimation is considered to be insignificant as soil GlcN content in the present study was 6 to 11 times greater than the soil MurN content in whole soil and fractions.

7.3.3 Statistical analyses

The data distribution was tested for normality by the Kolmogorov-Smirnov test and for homogeneity of variance by the Levene test. The effects of tillage and cropping systems were submitted to an analysis of variance (ANOVA; SAS ® v.9.4, Statistical Analysis System Institute, Cary, North Carolina). The statistical model in the analysis of variance to analyze amino-sugar concentration was: $Y_{ij} = \mu + B_i + T_j + Error_{(ij)}$ where μ is the general average of the experiment, B is block i (1, 2, 3), T represents treatment j (1, 2, 3, 4), and Error represents the experimental error. Treatment effects were tested using Tukey's test and declared significant at $P < 0.05$. When effects were significant, treatments means were compared by contrast analysis to determine the effect of:

i) Tillage effect: CT O/M 0N *vs*. NT O/M 0N;

ii) Legumes effect: NT O/M 0N *vs.* NT OV/MC 0N and NT OV/MC 180N;

iii) Fertilization effect: NT OV/MC 0N *vs.* NT OV/MC 180N.

Regression analyses were performed (SigmaPlot for Windows v. 13.0, Systat Software, Inc., San Jose, CA) to explore the relationships between GlcN or MurN concentrations taken individually or the GlcN/MurN ratio and previously published soil parameters such as aggregate mean weight diameter (MWD) and light-fraction C (Chapter 5 of this thesis). In addition, relationships between GlcN/MurN ratio and fine-silt and clay-C content, (Chapter 5 of this thesis), were also performed.

7.4 Results

The MurN concentration ranged from 3 to 20 mg kg^{-1} in the whole soil, and from 7 to 53 and 3 to 40 mg kg^{-1} in the clay and fine-silt fractions, respectively (Table 16). The greatest differences were recorded in the 0–5 cm soil layer. In whole soil, MurN concentration was 1.74 times greater in NT than in CT soils, 1.46 times greater with than without legume cover crops, and 1.14 times greater with than without N fertilization. The greatest effect of tillage was observed in the fine-silt fraction, where MurN concentration was more than two times greater under NT than CT. The MurN concentration was also 1.15 times greater with than without legume cover crops in the clay fraction. The positive effect of NT and legume cover crops was also observed in the whole soil in the 20–30 cm layer, whereas N fertilization decreased significantly MurN concentration in the fine-silt fraction. Treatments did not influence MurN concentration in the 75-100 cm soil layer.

Fungal-derived GlcN concentration ranged from 37 to 287 mg kg^{-1} in the whole soil, and from 63 to 451 and 21 to 597 mg kg^{-1} in the clay and fine-silt fractions, respectively (Table 16). Fungal-derived GlcN concentration generally showed trends similar to MurN, with the greatest treatment effects found in the whole soil of the 0–5 cm layer. In the whole soil, fungal GlcN was 2.11 times greater under NT than CT, 1.58 times greater with than without legume cover crops, and 1.23 times greater with than without N fertilization. In fine-silt fraction of the top soil layer, GlcN concentration was more than two times greater under NT than CT; in the clay fraction, GlcN concentration was 1.64 times greater under NT than CT, and 1.30 times greater with than without legumes cover crops. The positive effect

of NT and legume cover crops was also observed in the whole soil in the 20–30 cm layer. Nitrogen fertilization had no significant effect on GlcN at the 20–30 cm soil layer. Positive effects of tillage, legumes and N fertilization were found in the clay fraction of the 75–100 cm soil layer.

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Table 16. Muramic acid (MurN) fungal glusamine (GlucN) and GlucN/MurN ratio in whole soil and fine silt and clay fraction in 0–5, 20–30 e 75–100 cm layers from Acrisol submitted to conventional tillage (CT) and no tillage (NT) with oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC); and two nitrogen fertilization rates, 0 e 180 kg of urea ha^{-1} (0N e 180N).

Tillage	Cropping systems	N fertilization	Muramic acid			GlcN			GlucN/ MurN		
			Whole soil	Clay	Fine silt	Whole soil	Clay	Fine silt	WS	Clay	FS
			mg kg^{-1}								
			0–5 cm								
CT	O/M	0	7.04	30.85	11.77	78.45	208.12	142.58	11.15	6.79	12.39
NT	O/M	0	12.24	45.33	26.54	164.46	341.92	367.39	13.43	7.47	14.24
	OV/MC	0	16.65	50.69	37.85	232.43	439.03	525.69	13.95	8.72	13.99
	OV/MC	180	19.05	53.08	39.79	286.77	450.49	597.33	15.20	8.49	15.00
Contrast	CT vs NT		-5.21 *	-14.48**	-14.77**	-86.02**	-133.80*	-224.81**	-2.27*	-0.68	-1.84*
	OM vs OVMC		-11.20***	-13.12*	-24.57**	-190.27**	-205.69*	-388.24**	-2.30*	-2.26*	-0.51
	0 vs 180		-2.39*	-2.39	-1.94	-54.351**	-11.45	-71.63	-1.26	0.24	-1.02
			20 – 30 cm								
CT	O/M	0	4.83	16.89	8.21	53.01	120.95	73.90	11.11	7.33	9.14
NT	O/M	0	5.59	17.60	8.18	62.39	134.03	77.91	11.23	7.64	9.49
	OV/MC	0	6.28	18.60	10.88	72.02	139.16	98.17	11.50	7.63	9.01
	OV/MC	180	6.78	20.65	8.44	76.33	149.53	80.04	11.25	7.28	9.47
Contrast	CT vs NT		-0.77*	-0.71	0.03	-9.38*	-13.07*	-4.00	-0.13	-0.30	-0.35
	OM vs OVMC		-1.88*	-4.03	-2.96	-23.57**	-20.64*	-22.40	-0.27	0.36	0.50
	0 vs 180		-0.50	-2.05	2.44*	-4.31	-10.37	18.13	0.25	0.34	0.46
			75 – 100								
CT	O/M	0	2.71	6.46	3.74	36.46	63.44	27.24	13.56	10.11	7.26
NT	O/M	0	2.98	7.39	3.14	41.12	73.42	25.47	13.87	9.94	8.11
	OV/MC	0	3.14	7.06	3.51	44.17	76.98	28.67	14.04	10.96	8.22
	OV/MC	180	2.75	7.65	2.66	37.56	88.27	20.52	13.47	11.77	7.65
Contrast	CT vs NT		-0.27	-0.94	0.61	-4.69	-9.97*	1.76	-0.31	0.17	-0.85
	OM vs OVMC		0.08	0.07	-0.01	-0.50	-18.42*	1.75	0.25	-2.86	-0.36
	0 vs 180		0.38	-0.59	0.84	6.61	-11.28*	8.15	0.57	-0.81	0.58

Contrast analysis was used to test the effects of tillage (CT O/M 0 vs. NT O/M 0), legume cover cropping (CT O/M 0 vs. NT OV/MC 0 + NT OV/MC 180) and nitrogen fertilization (NT OV/MC 0 vs. NT OV/MC 180). Significant differences between treatments at 5%, 1% and <1 % probability of error by F test are designed with (*), (**) and (***), respectively

The GlcN/MurN ratio ranged from 11.1 to 15.2 in whole soil, and generally showed narrower values in the clay and fine-silt fractions, except for the fine-silt fraction in the 0-5 cm soil layer (Table 16). The GlcN/MurN ratio was affected by management system in the whole soil of the 0–5 cm soil layer, where it was greater under NT than CT, and greater with than without legumes. In that same soil layer, the GlcN/MurN ratio was greater under NT than CT in the fine-silt fraction, and greater with than without legumes in the clay fraction. Nitrogen fertilization had no effect on the GlcN/MurN ratio, and there were no treatment effects at the other soil depths. In the clay fraction, the GlcN/MurN ratio was greater at the 75–100 cm depth (10.7) than at 0-5 (7.9) and 20-30 (7.5) cm depths. By contrast, the GlcN/MurN ratio gradually declined with depth, from 13.9 to 7.8, in the fine-silt fraction.

Significant relationships were found between soil properties and amino-sugars in the 0–5 cm soil layer only and in the whole soil and clay- and fine-silt-sized fractions. Significant relationships between GlcN or MurN concentrations taken individually and LF-C (Fig. 23 a,b) and mean-weight diameter of water-stable soil aggregates (Fig 23 a,b) was observed. Besides, strong and positive relationships were found between the GlcN/MurN ratio and LF-C (Fig. 22c), and between the GlcN/MurN ratio and the mean-weight diameter of water-stable soil aggregates (Fig. 22c). In addition, positive relationships were observed between the GlcN/MurN ratio and C content in fine silt and clay fractions (Fig. 24) in the 0-5 cm soil layer only.

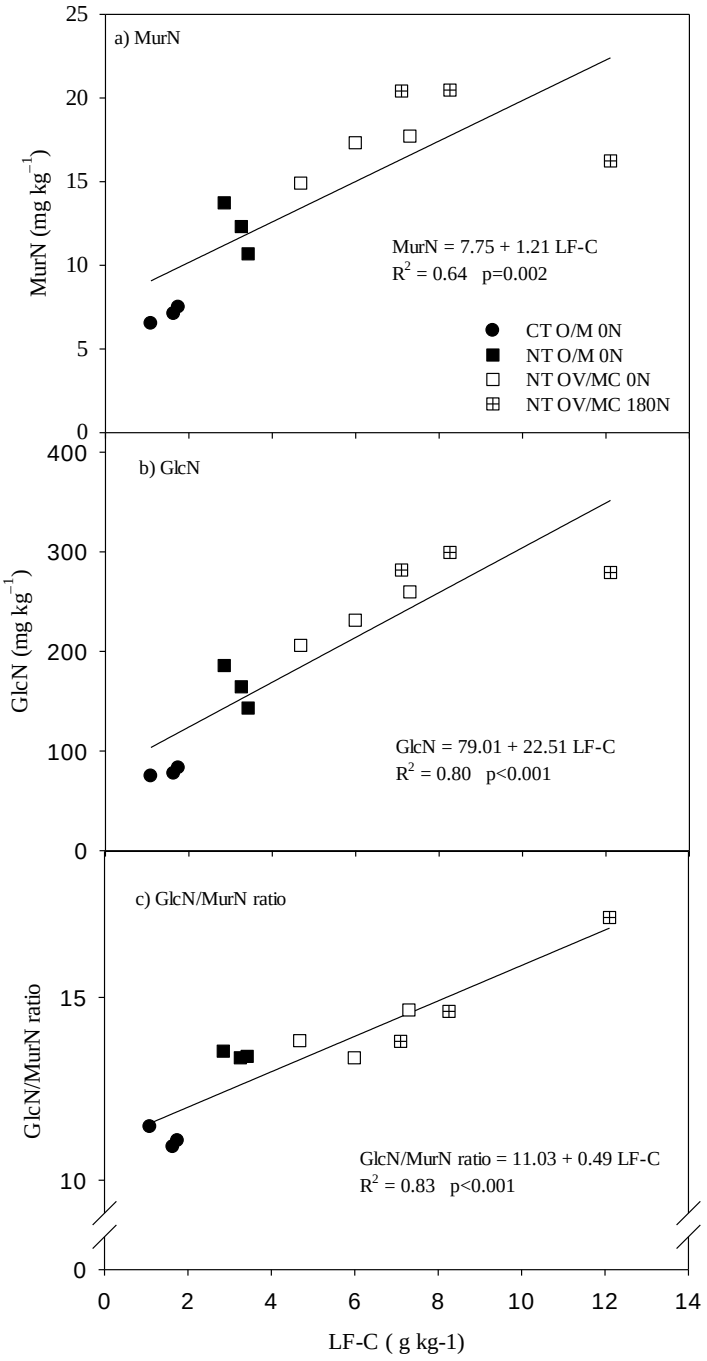

Figure 23. Regression of muramic acid concentration (a), glucosamine concentration (b) and glucosamine/muramic acid ratio (GlucN/MurN ratio) (c) as function of carbon content in light fraction (LF-C) of organic matter in the 0–5 cm soil deep layer from Acrisol submitted to conventional tillage (CT) and no tillage (NT) with oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC); and two nitrogen fertilization rates, 0 e 180 kg of urea ha^{-1} (0N e 180N).

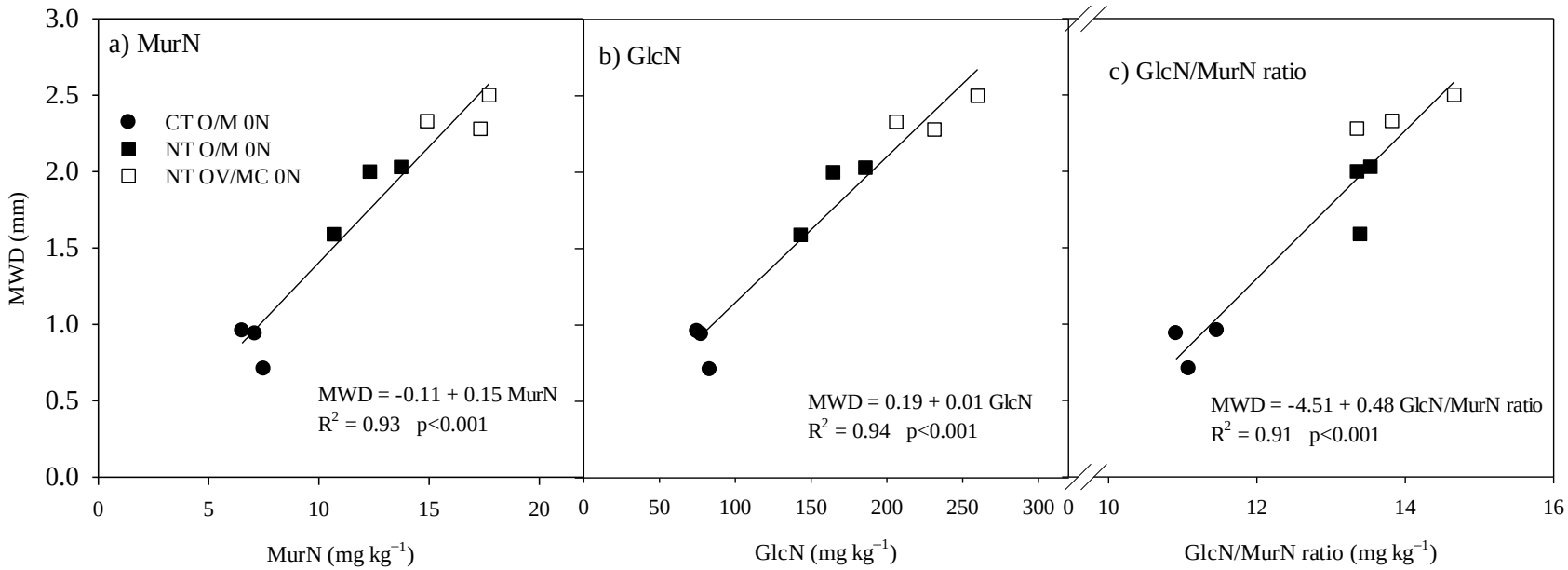

Figure 24. Regression of mean weighted diameter (MWD) of soil aggregates as a function of muramic acid concentration (a), glucosamine concentration (b) and glucosamine/muramic acid ratio (GlucN/MurN ratio) c) in the 0–5 cm soil deep layer from Acrisol submitted to conventional tillage (CT) and no tillage (NT) under oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC) cropping systems.

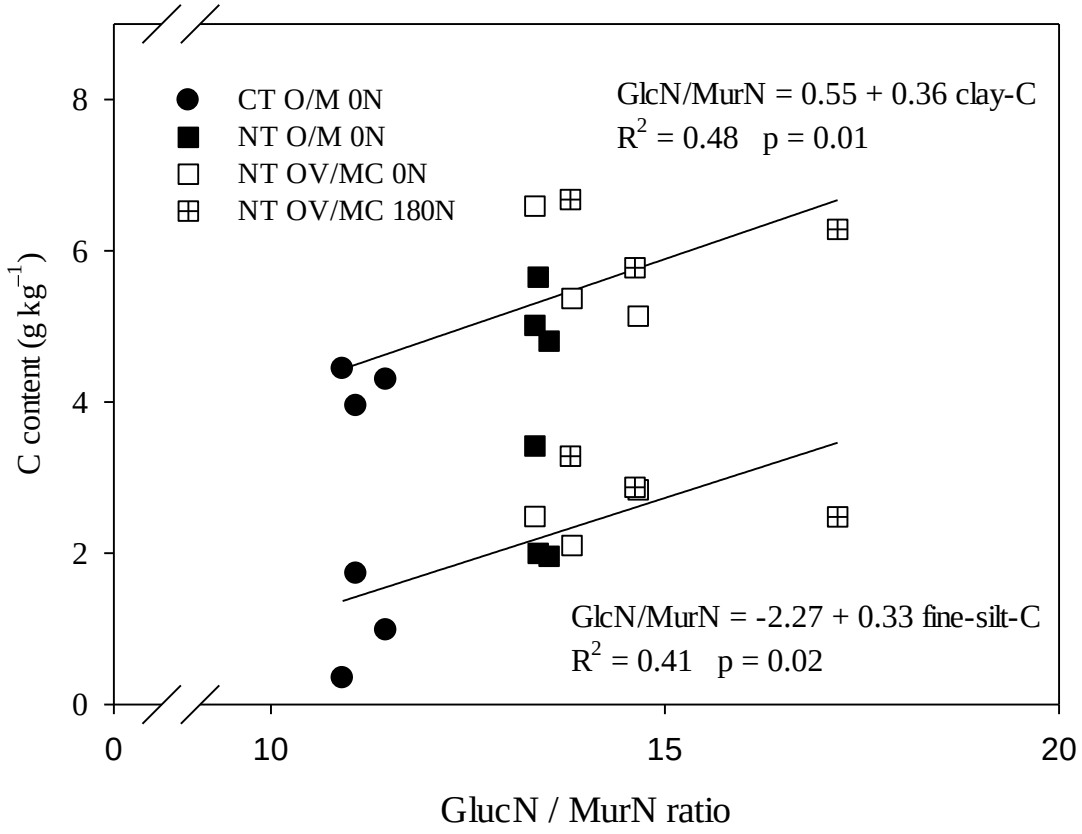

Figure 25. Regression of C content in fine-silt and caly fraction as a function of glucosamine/muramic acid ratio (GlucN/MurN ratio) in the 0–5 cm soil deep layer from Acrisol submitted to conventional tillage (CT) and no tillage (NT) under oat/maize (O/M) and oat+vetch/maize+cowpea (OV/MC) cropping systems and two nitrogen fertilization rates, 0 e 180 kg of urea ha^{-1} (0N e 180N).

7.5 Discussion

The long-term adoption of NT and legume cover crops increased soil macroaggregation and SOC content, specially the content of LF-C and clay-C at the 0–5 cm depth (Table 15). The MurN and GlcN concentrations measured in this experiment were similar to those reported by Martins et al. (2012b) under similar climate conditions. No-tillage, legume cover crops and N fertilization all favoured the co-accumulation of bacterial and fungal cell wall constituents in the whole soil, but the effects were mainly found in the top 5 cm of soil, and also to a

lower extent in the 20–30 cm layer for tillage and legume cover crops. This co-accumulation likely occurred because more substrates (e.g., greater C inputs through crop residues, and greater LF-C concentration; Table 15) were available for microorganisms in surface soil under NT, with legume cover crops, and with N fertilization. This is supported by the fact that microbial growth is generally mainly limited by the availability of labile C (Reinertsen et al., 1984; Bremer & van Kessel, 1992).

The mean GlcN/MurN ratio ranged from 6.8 to 15.2, consistent with values measured in top layers of a Brazilian Oxisol (Martins et al., 2012b). Besides the co-accumulation of bacterial and fungal cell wall constituents, tillage and legume cover cropping also significantly influenced the GlcN/MurN ratio. The greater GlcN/MurN ratio found under NT than CT indicates a preferential enrichment in fungal cell wall constituents under NT, in agreement with Frey et al. (1999). This preferential enrichment in fungal cell walls was likely due to greater C inputs from crop residues in the top 5 cm of soil (Table 15), which resulted in a marked accumulation of LF-C (Table 15). Accumulation of crop residues at or near the soil surface represents a good food source for microorganisms, and a favourable environment for extensive hyphal network development (Holland & Coleman, 1987), at the expense of bacteria which at less efficient than fungi at decomposing plant tissues (Griffiths et al., 1998; Boer et al., 2005).

The GlcN/MurN ratio was also significantly increased by the inclusion of legumes in the cropping system most likely because C inputs and LF-C accumulation were greater in cropping systems with than without legume cover crops (Table 15). In addition, living roots of legumes show greater root colonization by fungi than grasses (Scheublin et al., 2004). The influences of rooting patterns and densities should also be considered in GlcN enrichment since perennial legume showed significantly less amino sugars than perennial grasses, but annual legume (faba bean) showed more GlcN than annual grass species (wheat) (Chantigny et al., 1997).

Although N fertilization increased LF-C content compared to non-fertilized systems (Table 15), and GlcN and MurN concentrations in the top 5 cm of soil (whole soil only), there was no effect of N fertilization on either the GlcN/MurN ratio, indicating no preferential stimulation between either fungi or bacteria. Le

Guillou et al. (2012) also reported that N fertilization increased MurN and GlcN to similar extent in agricultural soils.

The strong and positive relationships found between the GlucN/MurN ratio and the mean-weight diameter of soil aggregates and LF-C concentration in the top 5 cm of soil suggest that the absence of physical soil disturbance under NT favoured the physical protection of LF in water-stable aggregates (Martins et al., 2012b; Conceição et al., 2013). Furthermore, soil aggregation around legume residues likely created an environment favourable to the colonization of LF by soil microbes (Six et al., 2002), and our results indicate that this environment was more favourable to the accumulation of fungal than bacterial cell wall constituents, as previously hypothesized by Martins et al. (2012b). That the relationship between GlcN/MurN ratio and soil aggregation was mostly limited to the top 5 cm of soil suggests that preferential accumulation of fungal components may be related to variations in soil moisture conditions, which are greater at the soil surface; it is recognized that fungi are more able than bacteria to cope with soil drying by crossing air-filled zones with their hyphae to access water and nutrients (Gordon et al., 2008; de Vries et al., 2012; Barnard et al., 2013). Increase in soil fungi populations under NT compared to CT have been documented previously, and were associated with increases in soil aggregation (Beare et al., 1997; Chantigny et al., 1997; Al-Maliki & Scullion, 2013), and more specifically in soil macroaggregates (Helgason et al., 2010). In turn, fungal hyphae improve aggregate resistance to mechanical breakdown and slaking (Abiven et al., 2009), and likely improved aggregate persistence in time.

For both amino-sugars, the highest concentration was found in the clay fraction which agrees with Simpson et al. (2004). In addition, we observed a significant positive effect of NT and legume cover crops on the accumulation of fungal glucosamine in the clay fraction down in the entire soil profile. This is consistent with findings in Chapter 5 of this thesis, who showed that C sequestration was likely related to microbial products accumulating at depth. Here we show that this is particularly true with fungal residues. This explains why the greater GlucN/MurN ratio in the clay fraction of deeper compared to superficial soil layers, suggesting an increase in the relative contribution of fungi over bacteria in deeper soil layers. It is possible that the low pH in deeper soil layers (the pH increases from 6.0 at 0–5 cm to 4.2 g kg^{-1} at 75–100 cm depth),

which is more favourable to fungal than bacterial growth (Rousk et al., 2009). Whether accumulation of GlcN at depth is caused by the migration of fungal cell-wall components or to the growth of fungi (e.g., colonization of roots by mycorrhizae) at depth remains to be ascertained. Compared to bacteria, fungi have a lower energy requirement and higher efficiency in transforming C substrates into microbial components, two factors likely to favour C accumulation in soil (Alvarez et al., 1995; Cotrufo et al., 2013; Kallenbach et al., 2016). Thus, differences in composition between bacterial and fungal communities are likely to influence soil C accumulation and stabilization (Fig. 25) over time. That the preferential accumulation of fungal-derived amino-sugars at depth was specifically found in the clay fraction suggests that these components are likely included in stable mineral-organic complexes.

7.6 Conclusions

Our results indicate that tillage and cropping systems are key factors driving physicochemical and microbiological properties of an Acrisol under subtropical climate. We emphasize that no tillage and legume cover cropping, favoured the co-accumulation of bacteria- and fungi-derived cell wall constituents in whole soil and in clay- and fine-silt-sized fractions. This co-accumulation was however limited to the top 5 cm of soil with a preferential enrichment of soil organic C in fungal-derived glucosamine, whereas fungal-derived glucosamine also accumulated down to 100 cm depth, and more specifically in the clay-sized fraction of soil. Our data suggest that (i) fungal growth is favoured by no tillage and legume cover crops which helps in stabilizing structural stability of surface soil and (ii) the accrual of fungal cell-wall residues is an important process in C accumulation and stabilization throughout the soil profile under subtropical climate.

8. SYNTHESIS RESULTS

Considering the results obtained in the research project for obtaining of doctorate degree, we formulated theoretical models that encompass the impact of no-tillage and legume cover crops on mechanisms of stabilization and accumulation of carbon in soil. The mineral-organic association was the main mechanism of carbon stabilization in this subtropical Acrisol. Greater contribution of mineral-organic association to soil organic carbon compared to other mechanisms was observed. In addition, the mineral-organic association was responsible for the long-term stabilization (hundred years). According to our results, there are two main paths favouring the mineral-organic association in agricultural systems: the labile residue input and favouring the soil aggregation.Residue type control residue-derived C and N partitioning among soil organic matter fractions. We hypothesize that the non-labile residue input is physically transferred to the soil and recovered as a light fraction. The light fraction can be stabilized promoting soil aggregation and be protected by spatial inaccessibility (Cotrufo et al., 2015; Martins & Angers, 2015). The soil aggregation is impacted mainly in superficial soil layers, because of residues are inputted on surface soil. The greater light fraction under no-tillage compared to conventional tillage is available for microrganisms in surface and favour the fungal community (fungal hyphae) which in turn mediated improvement of structural stability, specially the maccroaggregates. In large macroaggregates, the C occlusion appears to be the main mechanism of C stabilization, representing more than 70% of the MRT under NT and legume cover crops. In addition, the slower turnover of the macroaggregates under NT favour the C enrichment in microaggregates. Our study suggests that this especially the case when associated with legume cover crop, due to the importance of labile compounds in the formation of stable mineral-organic association.

The positive effect of high-quality residue such as legume cover crops in mineral-organic association was explained by favouring microbial processing. The greater contribution of microbial source to clay-C was observed more with than without legume cover crops, in surface and sub-surface soil layers, highlighting the role of soil microorganisms controlling the flow of C and N-residue to mineral-organic association. Some studies have shown that the low C/N ratio of substrate, such as legume, have favoured the carbon use efficiency by microorganisms (Manzoni et al., 2008). In addition, the legume cover crops favoured greater fungal community. Compared to bacteria, fungi have a lower energy requirement and higher efficiency in transforming C substrates into microbial components, two factors likely to favour C accumulation in soil (Alvarez et al., 1995; Cotrufo et al., 2013; Kallenbach et al., 2016). Thus, differences in composition between bacterial and fungal communities are likely to influence soil C accumulation and stabilization (Fig. 26) over time.

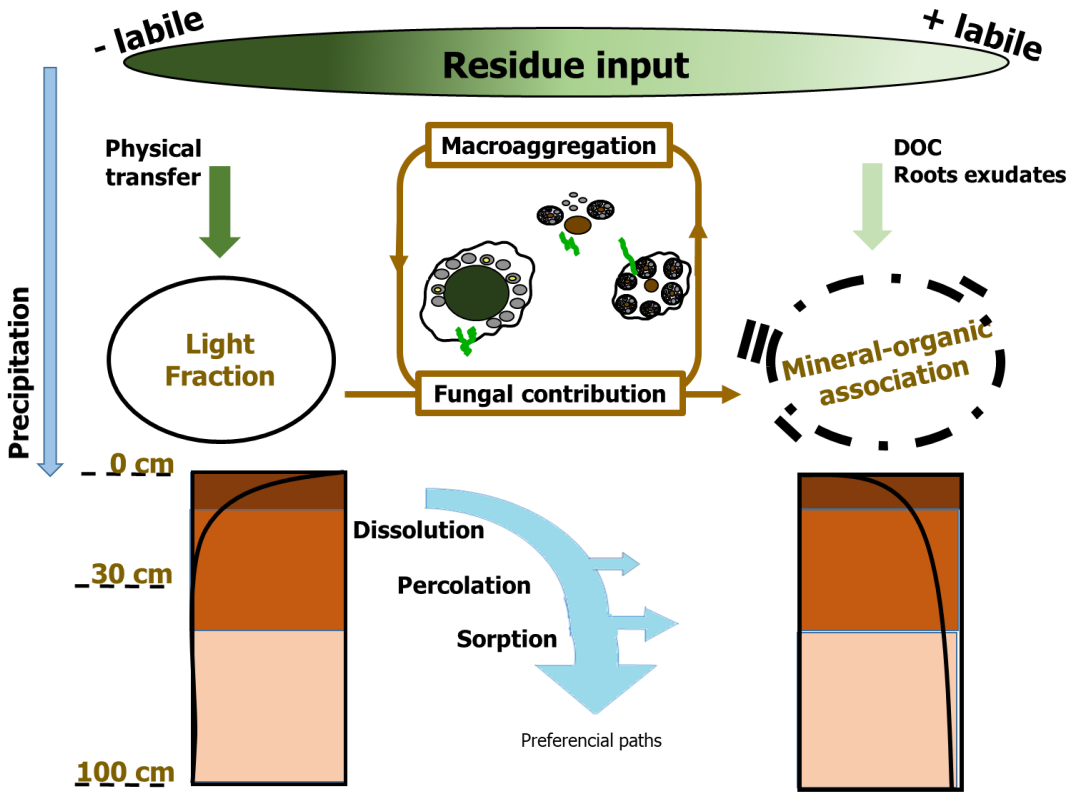

Figure 26. Theoretical model about stabilization and accumulation of carbon in soil profile.

In the sub-surface soil layer, the contribution of min-OM to SOC was greater compared to superficial soil layer (Fig 25). In that soil layers, NT and legume cover crops also favoured the C enrichment. The preferential

accumulation of fungal-derived amino-sugars at depth is linked to carbon isotope signature of cover crop residues at depth. The more negative values under legume cover crops compared to without legume cover crops in the surface and sub-surface layers are due to the greater contribution of residues of C3 plants (oats, vetch and cowpea). We believe that mean annual precipitation is an important factor to favour the leaching of soluble compounds to deeper soil layers. The high mean annual precipitation in subtropical site (1440 mm year^{-1}), taken together with the high residue input allied to the presence of biopores formed under NT possibly, favour the leaching of soluble compounds in soil profile that is adsorbed by mineral of sub-surface layers (Fig. 25). More than half of the C sequestration was attributable to the increase in C stocks in the sub-surface soil layer (30–100 cm), which add to the gains in C accumulation observed in 0–30 cm after 30 years adopting no-tillage and legume cover crops.

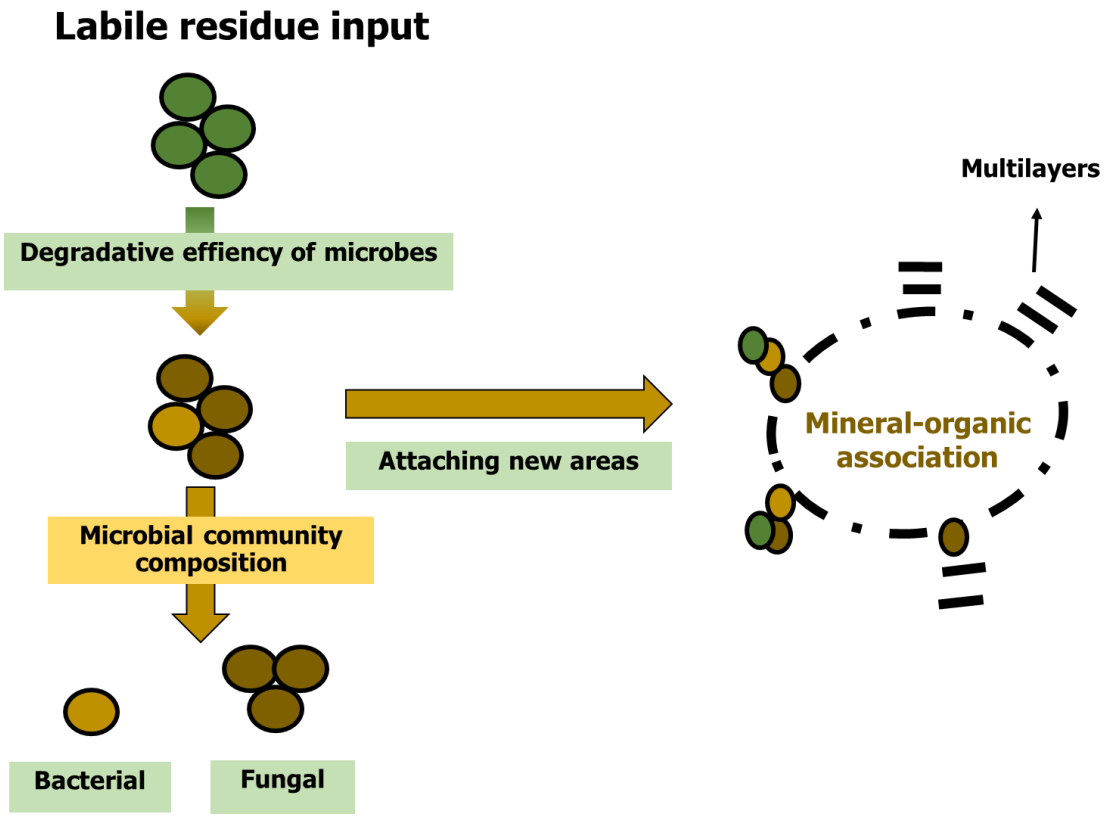

Figure 27. Conceptual model of carbon stabilization in mineral-organic associations from labile residue input considering the greater carbon use efficiency of soil fungal community.

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10. APENDICES

Apêndice 1. Densidade (g dm^{-3}) de um Argissolo Vermelho, em dois métodos de preparo e três sistemas de cultura, trinta anos após o início do experimento.

	Dose N	0 kg N ha^{-1} no milho								
Tratamentos	Camadas	Repetição			Média		Repetição			Média
		I	II	III			I	II	III	
PC A/M	**0 - 5**	1.52	1.51	1.43	1.49	PD A/M	1.43	1.44	1.40	1.42
	5 - 10	1.69	1.62	1.69	1.67		1.58	1.70	1.60	1.63
	10 - 15	1.72	1.73	1.69	1.71		1.62	1.75	1.61	1.66
	15 - 20	1.75	1.75	1.75	1.75		1.68	1.73	1.70	1.70
	20 - 30	1.70	1.72	1.71	1.71		1.57	1.69	1.56	1.61
	30 - 50	1.57	1.57	1.57	1.57		1.55	1.66	1.49	1.57
	50 - 75	1.50	1.53	1.53	1.52		1.49	1.58	1.43	1.50
	75 - 100	1.48	1.43	1.51	1.47		1.55	1.48	1.36	1.47
PC V/M	**0 - 5**	1.49	1.47	1.42	1.46	PD V/M	1.32	1.32	1.23	1.29
	5 - 10	1.68	1.70	1.61	1.67		1.52	1.59	1.55	1.55
	10 - 15	1.72	1.75	1.63	1.70		1.54	1.66	1.64	1.61
	15 - 20	1.73	1.74	1.68	1.72		1.57	1.68	1.68	1.64
	20 - 30	1.67	1.64	1.61	1.64		1.55	1.57	1.59	1.57
	30 - 50	1.59	1.52	1.61	1.57		1.52	1.54	1.49	1.52
	50 - 75	1.53	1.47	1.59	1.53		1.50	1.51	1.49	1.50
	75 - 100	1.46	1.44	1.51	1.47		1.41	1.49	1.44	1.44
PC A+V/M+C	**0 - 5**	1.53	1.44	1.54	1.50	PD A+V/M+C	1.27	1.15	1.20	1.21
	5 - 10	1.55	1.65	1.67	1.62		1.59	1.48	1.53	1.53
	10 - 15	1.62	1.68	1.68	1.66		1.63	1.61	1.62	1.62
	15 - 20	1.63	1.70	1.76	1.70		1.67	1.68	1.65	1.67
	20 - 30	1.53	1.62	1.69	1.61		1.60	1.60	1.61	1.60
	30 - 50	1.56	1.58	1.62	1.58		1.49	1.50	1.44	1.48
	50 - 75	1.51	1.54	1.50	1.52		1.45	1.49	1.47	1.47
	75 - 100	1.44	1.51	1.42	1.46		1.42	1.46	1.42	1.43

Apêndice 2. Teores de carbono orgânico total[1] ((g dm^{-3}) e densidade (DS) de um Argissolo Vermelho, em dois métodos de preparo e três sistemas de cultura, cinco anos após o início do experimento. (Bayer & Mielniczuk, 1997).

	Dose N	0 kg N ha^{-1} no milho					120 kg N ha^{-1} no milho				
Tratamentos	Camadas	Repetição			Média	DS	Repetição			Média	DS
		I	II	III			I	II	III		
PC A/M	0 - 2,5	1.09	1.12	1.05	1.09	1.52	1.31	1.17	1.04	1.17	1.52
	2,5 - 7	1.12	1.07	0.98	1.06	1.69	1.15	1.17	1.10	1.14	1.69
	7,5 - 17,5	1.20	1.17	0.99	1.12	1.72	1.21	1.11	1.06	1.13	1.72
PC A+T/M	0 - 2,5	1.21	1.13	1.10	1.15	1.52	1.19	1.17	1.29	1.22	1.52
	2,5 - 7	1.22	1.09	1.14	1.15	1.69	1.23	1.58	1.28	1.36	1.69
	7,5 - 17,5	1.05	0.90	1.16	1.04	1.72	0.95	0.90	1.06	0.97	1.72
PC A+T/M+C	0 - 2,5	1.22	1.23	1.13	1.19	1.52	1.27	1.26	1.10	1.21	1.52
	2,5 - 7	1.21	1.16	1.12	1.16	1.69	1.25	1.18	1.08	1.17	1.69
	7,5 - 17,5	0.95	0.92	0.86	0.91	1.72	1.04	0.98	0.94	0.99	1.72
PD A/M	0 - 2,5	1.39	1.78	1.39	1.52	1.52	1.57	1.64	1.62	1.61	1.52
	2,5 - 7	1.07	1.19	1.16	1.14	1.69	1.11	1.29	1.26	1.22	1.69
	7,5 - 17,5	0.82	1.02	0.88	0.91	1.72	0.08	0.09	1.05	0.41	1.72
PD A+T/M	0 - 2,5	1.63	2.00	1.62	1.75	1.52	2.04	2.16	2.21	2.14	1.52
	2,5 - 7	1.31	1.24	1.25	1.27	1.69	1.28	1.53	1.29	1.37	1.69
	7,5 - 17,5	0.09	0.10	0.09	0.09	1.72	0.94	0.99	0.94	0.96	1.72
PC A+T/M+C	0 - 2,5	1.82	2.24	1.92	1.99	1.52	1.97	1.99	1.95	1.97	1.52
	2,5 - 7	1.17	1.36	1.33	1.29	1.69	1.27	1.36	1.35	1.33	1.69
	7,5 - 17,5	0.84	0.91	0.95	0.90	1.72	0.92	1.02	0.87	0.94	1.72

Apêndice 3. Teores de carbono orgânico total[1] (g kg^{-1}) e densidade (DS) de um Argissolo Vermelho, em dois métodos de preparo e três sistemas de cultura, nove anos após o início do experimento.

Tratamentos	Dose N	0 kg N ha^{-1} no milho			Média	DS
	Camadas	Repetição				
		I	II	III		
PC A/M	0 - 2,5	16.70	16.90	15.60	16.40	1.44
	2,5 - 5	15.90	15.60	14.80	15.43	1.54
	5 - 7,5	15.70	16.90	14.20	15.60	1.54
	7,5 - 12,5	14.60	15.40	13.50	14.50	1.52
	12,5 - 17,5	15.10	15.40	14.60	15.03	1.52
	17,5 -30	14.40	14.40	14.40	14.40	1.62
PC A+T/M	0 – 2.5	17.70	16.40	17.00	17.03	
	2.5 - 5	15.60	14.80	16.70	15.70	
	5 – 7.5	15.90	15.10	16.20	15.73	
	7.5 – 12.5					
	12.5-17.5					
	17.5 -30					
PC A+T/M+C	0 - 2,5	17.50	20.70	16.60	18.27	1.44
	2.5 - 5	20.20	18.00	17.80	18.67	1.54
	5 – 7.5	18.30	17.20	16.10	17.20	1.54
	7.5 – 12.5	18.20	19.10	14.90	17.40	1.52
	12.5-17.,5	17.40	16.20	15.40	16.33	1.52
	17.5 -30	16.30	16.10	15.00	15.80	1.62
PD A/M	0 - 2,5	22.50	22.00	25.00	23.17	1.55
	2.5 - 5	19.00	1705.00	20.20	581.40	1.58
	5 – 7.5	15.70	15.40	17.30	16.13	1.58
	7.5 – 12.5	14.90	15.80	15.80	15.50	1.68
	12.5-17.,5	14.30	14.30	15.10	14.57	1.68
	17.5 -30	14.40	15.40	17.30	15.70	1.62
PD A+T/M	0 - 2,5	29.50	31.30	31.00	30.60	
	2.5 - 5	20.70	21.60	19.30	20.53	
	5 – 7.5	15.50	17.60	17.00	16.70	
	7.5 – 12.5					
	12.5-17.,5					
	17.5 -30					
PC A+T/M+C	0 - 2,5	36.40	34.80	35.90	35.70	1.55
	2.5 - 5	26.00	28.80	24.10	26.30	1.58
	5 – 7.5	19.30	21.80	19.60	20.23	1.58
	7.5 – 12.5	17.90	19.20	18.50	18.53	1.68
	12.5-17.,5	15.80	16.70	16.30	16.27	1.68
	17.5 -30	14.40	15.10	14.80	14.77	1.62

[1] Fonte: Bayer et al. (2000).

Apêndice 4. Teores de carbono orgânico total (g dm^{-3}) de um Argissolo Vermelho, em dois métodos de preparo e três sistemas de cultura, treze anos após o início do experimento. (Lovato et al., 2004)

Tratamentos	Dose N	0 kg N ha^{-1} no milho					180 kg N ha^{-1} no milho			
	Camadas	Repetição			Média	DS	Repetição			DS
		I	II	III			I	II	III	
PC A/M	0 - 2.5	1.20	1.12	1.26	1.19	1.44	1.45	1.40	1.44	1.43
	2.5 - 5	0.98	1.01	0.82	0.94	1.54	1.22	1.26	1.17	1.22
	5 - 7.5	1.05	1.05	0.81	0.97	1.54	1.04	1.08	0.97	1.03
	7.5 - 12.5	0.96	0.86	0.99	0.94	1.52	1.07	1.12	1.03	1.07
	12.5 - 17.5	0.94	1.03	0.84	0.94	1.52	1.12	1.08	0.93	1.04
	17.5 -30	1.00	0.94	0.89	0.94	1.62	0.96	1.16	1.10	1.07
PC V/M	0 - 2.5	1.51	1.49	1.50	1.50	1.44	1.32	1.48	1.53	1.44
	2.5 - 5	1.02	1.08	1.03	1.04	1.54	1.17	1.35	1.23	1.25
	5 - 7.5	1.03	1.05	1.05	1.04	1.54	1.12	1.22	1.22	1.19
	7.5 - 12.5	0.92	1.01	0.96	0.96	1.52	1.12	1.12	1.21	1.15
	12.5 - 17.5	1.00	0.93	0.89	0.94	1.52	0.93	1.19	1.14	1.09
	17.5 -30	0.95	0.99	0.95	0.96	1.62	0.90	1.08	1.10	1.03
PC A+V/M+C	0 - 2.5	1.47	1.68	1.37	1.51	1.44	1.76	1.58	1.34	1.56
	2.5 - 5	1.37	1.15	1.20	1.24	1.54	1.28	1.30	1.14	1.24
	5 - 7.5	1.26	0.98	1.13	1.12	1.54	1.17	1.12	1.08	1.12
	7.5 - 12.5	1.15	0.92	1.13	1.07	1.52	0.99	0.99	0.91	0.96
	12.5 - 17.5	0.99	0.90	1.12	1.00	1.52	1.70	1.52	1.56	1.59
	17.5 -30	0.97	0.96	0.94	0.96	1.62	1.41	1.30	1.37	1.36
PD A/M	0 - 2.5	1.73	1.95	1.55	1.74	1.55	1.99	1.92	2.05	1.99
	2.5 - 5	1.38	1.26	1.12	1.25	1.58	1.32	1.43	2.04	1.60
	5 - 7.5	1.08	1.16	0.98	1.07	1.58	1.10	1.01	1.17	1.09
	7.5 - 12.5	1.05	1.10	0.95	1.03	1.68	0.93	1.04	0.98	0.98
	12.5 - 17.5	1.00	0.98	0.88	0.95	1.68	0.82	1.08	1.05	0.98
	17.5 -30	0.90	0.95	0.98	0.94	1.62	0.82	1.13	1.01	0.99
PD V/M	0 - 2.5	1.93	2.21	2.19	2.11	1.55	1.98	2.40	2.42	2.27
	2.5 - 5	1.18	1.67	1.50	1.45	1.58	1.77	1.79	1.46	1.67
	5 - 7.5	1.08	1.21	1.10	1.13	1.58	1.37	1.37	1.14	1.29
	7.5 - 12.5	0.98	1.03	1.05	1.02	1.68	1.01	1.10	0.92	1.01
	12.5 - 17.5	0.97	1.04	0.90	0.97	1.68	0.98	0.98	1.02	0.99
	17.5 -30	0.79	1.06	0.91	0.92	1.62	1.01	0.97	1.11	1.03
PD A+V/M+C	0 - 2.5	2.25	2.48	1.96	2.23	1.55	2.31	2.71	2.70	2.57
	2.5 - 5	1.68	1.68	1.58	1.65	1.58	1.76	1.78	1.86	1.80
	5 - 7.5	1.37	1.19	1.26	1.27	1.58	1.35	1.32	1.47	1.38
	7.5 - 12.5	1.13	1.03	1.05	1.07	1.68	1.14	1.04	1.00	1.06
	12.5 - 17.5	1.02	1.00	1.05	1.02	1.68	0.98	0.99	0.96	0.98
	17.5 -30	0.94	0.94	0.95	0.94	1.62	0.94	1.02	0.92	0.96

Apêndice 5. Teores de carbono orgânico total (g kg^{-1}) de um Argissolo Vermelho, em dois métodos de preparo e três sistemas de cultura, dezoito anos após o início do experimento. *(*Zanatta et al., 2007).

	Dose N	0 kg N ha^{-1} no milho					180 kg N ha^{-1} no milho			
Tratamentos	Camadas	Repetição			Média	DS	Repetição			Média
		I	II	III			I	II	III	
PC A/M	0 -2.5	9.72	10.02	9.10	9.61	1.44	9.38	10.53	10.75	10.22
	2.5-5.0	9.09	8.30	8.32	8.57	1.54	9.25	10.31	9.58	9.71
	5-10	9.01	8.55	8.89	8.82	1.54	8.95	9.69	8.94	9.19
	10-20	8.68	9.00	8.16	8.61	1.52	8.98	9.35	9.71	9.35
	20-30	9.11	8.57	8.19	8.62	1.62	9.59	9.43	9.33	9.45
PC V/M	0 -2.5	10.56	11.11	10.65	10.77	1.44	10.16	10.38	11.89	10.81
	2.5-5.0	9.43	9.44	11.04	9.97	1.54	10.46	9.24	11.22	10.31
	5-10	9.87	10.04	10.17	10.03	1.54	10.58	9.21	9.81	9.87
	10-20	9.39	9.96	10.01	9.79	1.52	9.95	9.41	10.74	10.03
	20-30	9.69	9.17	9.93	9.60	1.62	9.80	9.40	10.49	9.90
PC A+V/M+C	0 -2.5	12.83	13.11	9.76	11.90	1.44	13.02	12.48	12.91	12.80
	2.5-5.0	10.86	11.22	9.94	10.67	1.54	11.63	11.53	10.82	11.33
	5-10	11.47	10.54	9.61	10.54	1.54	10.50	10.77	10.13	10.47
	10-20	10.94	9.55	9.09	9.86	1.52	9.85	10.78	9.88	10.17
	20-30	9.90	9.59	8.99	9.49	1.62	9.40	9.86	8.93	9.40
PD A/M	0 -2.5	14.94	16.23	18.17	16.45	1.55	19.44	19.75	20.48	19.89
	2.5-5.0	10.17	12.14	12.42	11.58	1.58	15.85	13.30	13.69	14.28
	5-10	8.72	9.14	8.61	8.82	1.58	9.41	9.42	9.95	9.59
	10-20	8.50	9.30	8.50	8.77	1.68	7.88	8.46	8.10	8.15
	20-30	9.21	9.10	10.07	9.46	1.62	8.47	8.40	10.10	8.99
PD V/M	0 -2.5	21.86	21.55	20.23	21.21	1.55	22.90	20.81	16.38	20.03
	2.5-5.0	13.10	18.04	14.43	15.19	1.58	17.02	14.08	11.29	14.13
	5-10	9.69	11.73	10.25	10.56	1.58	11.02	10.57	10.16	10.58
	10-20	8.27	9.14	8.77	8.73	1.68	8.41	8.37	8.63	8.47
	20-30	8.45	9.25	8.87	8.86	1.62	8.84	9.13	8.87	8.95
PD A+V/M+C	0 -2.5	21.30	22.41	22.79	22.17	1.55	19.70	28.50	24.70	24.30
	2.5-5.0	14.33	15.74	17.59	15.89	1.58	14.28	18.45	17.46	16.73
	5-10	9.58	10.65	11.79	10.67	1.58	10.25	12.30	12.51	11.69
	10-20	8.27	8.71	10.17	9.05	1.68	9.19	8.81	9.48	9.16
	20-30	8.79	9.04	10.36	9.40	1.62	8.76	9.89	9.99	9.55

Apêndice 6. Teores de carbono orgânico total (g kg^{-1}) de um Argissolo Vermelho, em dois métodos de preparo e três sistemas de cultura, trinta anos após o início do experimento, usados neste estudo.

	Dose N	0 kg N ha^{-1} no milho					180 kg N ha^{-1} no milho			
Tratamentos	Camadas	Repetição			Média	DS	Repetição			Média
		I	II	III			I	II	III	
PC A/M	0 -5	9.98	12.29	9.41	10.56	1.49	12.20	11.09	10.58	11.29
	5-10	9.21	9.98	9.17	9.45	1.67	10.79	10.09	9.19	10.02
	10-15	10.57	8.82	8.93	9.44	1.71	12.45	10.53	8.80	10.59
	15-20	10.01	9.35	9.89	9.75	1.75	9.48	9.87	8.90	9.42
	20-30	9.22	9.03	9.07	9.11	1.71	11.25	9.97	9.89	10.37
PC V/M	0 -5	13.20	10.46	12.95	12.20	1.49	13.42	14.98	14.02	14.14
	5-10	12.26	11.58	12.02	11.95	1.67	12.96	10.72	12.54	12.07
	10-15	10.42	11.16	10.89	10.82	1.71	10.89	9.68	10.67	10.41
	15-20	9.61	10.37	9.95	9.98	1.75	9.83	8.92	8.97	9.24
	20-30	9.42	9.78	10.37	9.86	1.71	9.86	9.59	10.19	9.88
PC A+V/M+C	0 -5	13.49	15.00	11.64	13.38	1.49	10.50	15.16	13.05	12.91
	5-10	11.47	12.01	11.74	11.74	1.67	11.13	12.23	10.11	11.16
	10-15	12.95	13.65	11.14	12.58	1.71	11.59	12.27	11.53	11.80
	15-20	11.51	10.75	9.41	10.56	1.75	10.25	10.59	10.05	10.30
	20-30	10.75	9.62	9.60	9.99	1.71	10.88	10.11	10.33	10.44
PD A/M	0 -5	15.12	14.17	15.61	14.97	1.49	17.00	17.60	17.11	17.24
	5-10	8.53	8.63	9.67	8.94	1.67	9.93	9.68	9.75	9.79
	10-15	8.33	8.24	9.79	8.79	1.71	8.76	10.40	8.32	9.16
	15-20	8.75	7.82	9.67	8.75	1.75	8.72	8.38	8.95	8.68
	20-30	9.05	9.20	10.01	9.42	1.71	10.32	9.84	10.12	10.10
PD V/M	0 -5	18.43	20.43	19.43	19.43	1.49	21.71	22.80	24.09	22.87
	5-10	10.74	10.97	9.81	10.50	1.67	10.49	13.38	12.05	11.97
	10-15	8.73	9.35	8.39	8.82	1.71	8.73	9.81	8.79	9.11
	15-20	7.81	8.63	8.72	8.39	1.75	8.58	7.14	9.00	8.24
	20-30	9.79	9.22	10.30	9.77	1.71	9.71	10.67	10.49	10.29
PD A+V/M+C	0 -5	18.78	20.35	23.45	20.86	1.49	24.69	29.30	23.68	25.89
	5-10	10.96	12.12	11.97	11.68	1.67	12.66	12.81	14.34	13.27
	10-15	8.79	10.02	10.49	9.76	1.71	9.55	9.99	9.78	9.78
	15-20	8.64	9.52	9.90	9.35	1.75	9.16	9.00	8.32	8.83
	20-30	10.30	10.59	10.86	10.58	1.71	10.15	9.82	10.07	10.02